Printed: 20-04-2005 TIPA1 99910592

To The European Patent Office

15 April 2005

Application No. 99910592.7
COMMONWEALTH SCIENTIFIC AND
INDUSTRIAL RESEARCH ORGANISATION

These comments are filed as third party observations under Article 115 EPC.

The Supplementary European Search Report recently issued in connection with the above-mentioned application. However, a number of relevant documents were not cited in that Supplementary Search Report or in the International Search Report that previously issued from the Australian Patent Office. We therefore wish to bring the Examiner's attention to a number of further documents which are relevant to the patentability of the present case.

In particular, the following documents are citable against the above mentioned application under Article 54(3) EPC:

- WO 9932619 (Carnegie Institution of Washington and The University of Massachusetts);
- WO 9949029 (AG-Gene Australia Limited and State of Queensland through its Department of Primary Industries); and
- WO 9853083 (Syngenta Limited).

Each of these PCT applications has been brought into the European regional phase, and a copy of each is enclosed. Their priority documents are also enclosed.

We would also like to inform the Examiner of the following documents, which are citable against the above-mentioned application under Article 54(2) and Article 56 EPC:

- Dorer, D and Henikoff, S, *Cell* 77(7): 993-1002 (1994);
- Sijen, T *et al.*, *The Plant Cell* 8: 2277-2294 (1996);

EPO - DG 1
20. 04. 2005
(93)

Best Available Copy

20-04-2005

- Jorgensen, R *et al.*, *Mol. Gen. Genet.* 207: 471-477 (1987);
- WO 97/01952 (DNA Plant Technology Corporation);
- WO 94/01550 (Hybridon Inc.);
- Kuipers, A *et al.*, *Mol. Gen. Genet.* 246: 745-755 (1995);
- Hergersberg M, Inaugural-Dissertation, Universität Köln (1998);
- "Safety Assessment of Genetically Engineered Fruits and Vegetables - A Case Study of the FlavrSavr™ Tomato", CRC Press (1992); and
- Van Blokland, R *et al.*, Post-transcriptional suppression of chalcone synthase genes in *Petunia hybrida* and the accumulation of unspliced pre-mRNAs, Mechanisms and Applications of Gene Silencing, 57-69, Grierson *et al.* (Eds), Nottingham University Press (1996).

A copy of each of these documents is also enclosed.

Further, a document by:

- Fire, A *et al.* (*Nature* 391: 806-811 (1998))

that was cited in the International Search Report in category A is in fact pertinent to the inventive step of the above-mentioned application and is more relevant than its categorization in the International Search Report indicates.

We would also like the Examiner to be aware of the fact that nine oppositions have been filed against European patent EP-B1-0983370 resulting from WO 9853083 (Syngenta Limited), mentioned above. A consultation of the EPO's file for that patent reveals a large number of prior art documents that are also potentially relevant to the present case.

Additionally, eight oppositions have been filed against a patent by Ribopharma AG (EP-B1-1144623) that is also in this technical field. The EPO's file for that case also contains prior art that is potentially relevant to the present case.

Lastly, relevant prior art is also contained in the EPO's files relating to examination of the three Article 54(3) documents mentioned above.

Printed: 22-04-2005 TIPA1 99910592

EPO - DG 1
01. 08. 2000
(43)

REC'D 28 JAN 1999
WIPO PCT

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

January 22, 1999

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/068,562*
FILING DATE: *December 23, 1997*
PCT APPLICATION NUMBER: *PCT/US98/27233*

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

MARGARET BASSFORD
Certifying Officer

57

20-04-2005

A/Prov

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

REQUEST FOR FILING PROVISIONAL PATENT APPLICATION

Under 35 USC 111(b)

(Not for DESIGN cases)

Box: PROVISIONAL APPLICATION

12/23/97 U.S. PTO

Commissioner of Patents
Washington, D.C. 20231

PROVISIONAL APPLICATION
Under Rule 53(c)

Sir:

(Our Deposit Account No. 03-3975)

Herewith is a PROVISIONAL APPLICATION
Title: GENETIC INHIBITION BY DOUBLE-STRANDED RNA

Our Order No. 20263 (C#) | 243343 (M#)

Atty. Dkt. PMS 243343 (M#) | Client Ref

Date: December 23, 1997

including:

1. Specification: 29 pages 2. ☐ Specification in non-English language 3. ☒ Drawings: 4 sheet(s)
4. The invention ☒ was ☐ was not made by, or under a contract with, an agency of the U.S. Government.
 If yes, Government agency/contact # = NIH/#GM-37706, #GM-17164, #HD-33769 and #GM-07231
5. ☐ Attached is an assignment and cover sheet. Please return the recorded assignment to the undersigned.
6. ☐ Attached: _____ (No.) Verified Statement(s) establishing "small entity" status under Rules 9 & 27.
 NOTE: Do NOT File IDS!
7. ☐ Attached:

8. This application is made by the following named inventor(s) **(Double check instructions for accuracy.):**

	First	Middle Initial	Family Name
(1) Inventor	Andrew		Fire
Residence	Baltimore (City)	Maryland (State/Foreign Country)	U.S.A. (Country of Citizenship)

	First	Middle Initial	Family Name
(2) Inventor	Si	Q.	Xu
Residence	Baltimore (City)	Maryland (State/Foreign Country)	China (Country of Citizenship)

	First	Middle Initial	Family Name
(3) Inventor	Mary	F.	Montgomery
Residence	Baltimore (City)	Maryland (State/Foreign Country)	U.S.A. (Country of Citizenship)

	First	Middle Initial	Family Name
(4) Inventor	Stephen	A.	Kostas
Residence	Baltimore (City)	Maryland (State/Foreign Country)	U.S.A. (Country of Citizenship)

	First	Middle Initial	Family Name
(5) Inventor	Samuel	E.	Driver
Residence	Cambridge (City)	Massachusetts (State/Foreign Country)	U.S.A. (Country of Citizenship)

9. NOTE: FOR ADDITIONAL INVENTORS, check box ☒ and attach sheet (PAT102A3) with same information regarding additional inventors.

REQUEST FOR FILING APPLICATION
Under 35USC 111(b)
(Continued : Additional Inventors)

Inventor	Craig	C.	Mello	
	First	Middle Initial	Family Name	
Residence	Worcester	Massachusetts	U.S.A.	
	City	State/Foreign Country	Country of Citizenship	

(7) Inventor				
	First	Middle Initial	Family Name	
Residence				
	City	State/Foreign Country	Country of Citizenship	

(8) Inventor				
	First	Middle Initial	Family Name	
Residence				
	City	State/Foreign Country	Country of Citizenship	

(9) Inventor				
	First	Middle Initial	Family Name	
Residence				
	City	State/Foreign Country	Country of Citizenship	

(10) Inventor				
	First	Middle Initial	Family Name	
Residence				
	City	State/Foreign Country	Country of Citizenship	

(11) Inventor				
	First	Middle Initial	Family Name	
Residence				
	City	State/Foreign Country	Country of Citizenship	

(12) Inventor				
	First	Middle Initial	Family Name	
Residence				
	City	State/Foreign Country	Country of Citizenship	

(13) Inventor				
	First	Middle Initial	Family Name	
Residence				
	City	State/Foreign Country	Country of Citizenship	

(14) Inventor				
	First	Middle Initial	Family Name	
Residence				
	City	State/Foreign Country	Country of Citizenship	

PAT102A3 12/97

Page 1A

	Large/Small Entity		Fee Code
Filing Fee	$150/$75	+150	114/214
11. If "non-English" box 2 is X'd, add Rule 17(k) processing fee	$130	+	139
12. If "assignment" box 5 is X'd, add recording fee	$40	+	581
13. TOTAL FEE ENCLOSED =	$150		

CHARGE STATEMENT: The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-17 (missing or insufficient fee only) now or hereafter relative to this application or credit any overpayment, to our Account/Order Nos. shown in the heading hereof for which purpose a duplicate copy of this sheet is attached.

Pillsbury Madison & Sutro LLP
Intellectual Property Group
1100 New York Avenue, N.W.
Ninth Floor East Tower
Washington, D.C. 20005-3918
Tel: (202) 861-3000
By: Atty: Paul N. Kokulis Reg. No. 16,773
Sig:
Fax: (202) 822-0944
Tel: (202) 861-3503
Atty/Sec: PNK/GRT/maf

NOTE: File in duplicate with 2 post card receipts (PAT-103) & attachments

09003562.PNK

APPLICATION UNDER UNITED STATES PATENT LAWS

Invention: GENETIC INHIBITION BY DOUBLE-STRANDED RNA

Inventor (s): Andrew FIRE, Si Q. XU, Mary K. MONTGOMERY, Stephen A. KOSTAS, Samuel E. DRIVER, and Craig C. MELLO

Pillsbury Madison & Sutro LLP
Intellectual Property Group
1100 New York Avenue, N.W.
Ninth Floor, East Tower
Washington, D.C. 20005-3918
Attorneys
Telephone: (202) 861-3000

Express Mail Label No. EL 990105910 US

This is a:

- ☒ Provisional Application
- ☐ Regular Utility Application
- ☐ Continuing Application
- ☐ PCT National Phase Application
- ☐ Design Application
- ☐ Reissue Application
- ☐ Plant Application
- ☐ Substitute Specification
 Sub. Spec Filed _____
 in App. No. _/_____

SPECIFICATION

GENETIC INHIBITION BY DOUBLE-STRANDED RNA

GOVERNMENT RIGHTS

This invention was made with U.S. government support under grant numbers GM-37706, GM-17164, HD-33769 and GM-07231 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to gene-specific inhibition of gene expression by double-stranded ribonucleic acid (dsRNA).

2. Description of the Related Art

Targeted inhibition of gene expression has been a long-felt need in biotechnology and genetic engineering. Although a major investment of effort has been made to achieve this goal, a more comprehensive solution to this problem was still needed.

Classical genetic techniques have been used to isolate mutant organisms with reduced expression of selected genes. Although valuable, such techniques require laborious mutagenesis and screening programs, are limited to organisms in which genetic manipulation is well established (e.g., the existence of selectable markers, the ability to control genetic segregation and sexual reproduction), and are limited to applications in which a large number of cells or organisms can be sacrificed to isolate the desired mutation. Even under these circumstances, classical genetic techniques can fail to produce mutations in specific target genes of interest, particularly when complex genetic pathways are involved. Many applications of molecular genetics require the ability to go beyond classical genetic screening techniques and efficiently produce a *directed* change in gene expression in a specified group of cells or organisms. Some such applications are knowledge-based projects in which it is of importance to understand what effects the loss of a specific gene product (or products) will have on the behavior of the cell or organism. Other applications are engineering based: for example, cases in which is important to produce a population of cells or organisms in which a specific gene product (or products) has been reduced or removed. A further class of applications is therapeutically based in which it would be valuable for a functioning organism (e.g., a human) to reduce or remove the amount of a specified gene product (or products). Another class of

EP0360882 - 20-04-2005

applications provides a disease model in which a physiological function in a living organism is genetically manipulated to reduce or remove a specific gene product (or products) without making a permanent change in the organism's genome.

In the last few years, advances in nucleic acid chemistry and gene transfer have inspired new approaches to engineer specific interference with gene expression. These approaches are described below.

Use of Antisense Nucleic Acids to Engineer Interference

Antisense technology has been the most commonly described approach in protocols to achieve gene-specific interference. For antisense strategies, stochiometric amounts of single-stranded nucleic acid complementary to the messenger RNA for the gene of interest are introduced into the cell. Some difficulties with antisense-based approaches relate to delivery, stability, and dose requirements. In general, cells do not have an uptake mechanism for single-stranded nucleic acids, hence uptake of unmodified single-stranded material is extremely inefficient. While waiting for uptake into cells, the single-stranded material is subject to degradation. Because antisense interference requires that the interfering material accumulate at a relatively high concentration (at or above the concentration of endogenous mRNA), the amount required to be delivered is a major constraint on efficacy. As a consequence, much of the effort in developing antisense technology has been focused on the production of modified nucleic acids that are both stable to nuclease digestion and able to diffuse readily into cells. The use of antisense interference for gene therapy or other whole-organism applications has been limited by the large amounts of oligonucleotide that need to be synthesized from non-natural analogs, the cost of such synthesis, and the difficulty even with high doses of maintaining a sufficiently concentrated and uniform pool of interfering material in each cell.

Triple-Helix Approaches to Engineer Interference

A second, proposed method for engineered interference is based on a triple helical nucleic acid structure. This approach relies on the rare ability of certain nucleic acid populations to adopt a triple-stranded structure. Under physiological conditions, nucleic acids are virtually all single- or double-stranded, and rarely if ever form triple-stranded

structures. It has been known for some time, however, that certain simple purine- or pyrimidine-rich sequences could form a triple-stranded molecule *in vitro* under extreme conditions of pH (i.e., in a test tube). Such structures are generally very transient under physiological conditions, so that simple delivery of unmodified nucleic acids designed to produce triple-strand structures does not yield interference. As with antisense, development of triple-strand technology for use *in vivo* has focused on the development of modified nucleic acids that would be more stable and more readily absorbed by cells *in vivo*. An additional goal in developing this technology has been to produce modified nucleic acids for which the formation of triple-stranded material proceeds effectively at physiological pH.

Co-Suppression Phenomena and Their Use in Genetic Engineering

A third approach to gene-specific interference is a set of operational procedures grouped under the name "co-suppression". This approach was first described in plants and refers to the ability of transgenes to cause silencing of an unlinked but homologous gene. More recently, phenomena similar to co-suppression have been reported in two animals: *C. elegans* and *Drosophila*. Co-suppression was first observed by accident, with reports coming from groups using transgenes in attempts to achieve over-expression of a potentially useful locus. In some cases the over-expression was successful while, in many others, the result was opposite from that expected. In those cases, the transgenic plants actually showed less expression of the endogenous gene. Several mechanisms have so far been proposed for transgene-mediated co-suppression in plants; all of these mechanistic proposals remain hypothetical, and no definitive mechanistic description of the process has been presented. The models that have been proposed to explain co-suppression can be placed in two different categories. In one set of proposals, a direct physical interaction at the DNA- or chromatin-level between two different chromosomal sites has been hypothesized to occur; an as-yet-unidentified mechanism would then lead to *de novo* methylation and subsequent suppression of gene expression. Alternatively, some have postulated an RNA intermediate, synthesized at the transgene locus, which might then act to produce interference with the endogenous gene. The characteristics of the interfering

EP0603354A2

RNA, as well as the nature of the interference process, have not been determined. Recently, a set of experiments with RNA viruses have provided some support for the possibility of RNA intermediates in the interference process. In these experiments, a replicating RNA virus is modified to include a segment from a gene of interest. This modified virus is then tested for its ability to interfere with expression of the endogenous gene. Initial results with this technique, presented in the literature over the last few months, have been encouraging. However the properties of the viral RNA that are responsible for interference effects have not been determined and, in any case, would be limited to plants which are hosts of the plant virus.

Distinction between the Present Invention and Antisense Approaches

The present invention differs from antisense-mediated interference in both approach and effectiveness. Antisense-mediated genetic interference methods have a major challenge: delivery to the cell interior of specific single-stranded nucleic acid molecules at a concentration that is equal to or greater than the concentration of endogenous mRNA. Double-stranded RNA-mediated inhibition has advantages both in the stability of the material to be delivered and the concentration required for effective inhibition. Below, we disclose that in the model organism *C. elegans*, the present invention is at least 100-fold more effective than an equivalent antisense approach (i.e., dsRNA is at least 100-fold more effective than the injection of purified antisense RNA in reducing gene expression). These comparisons also demonstrate that inhibition by double-stranded RNA must occur by a mechanism distinct from antisense interference.

Distinction between the Present Invention and Triple-Helix Approaches

The limited data on triple strand formation argues against the involvement of a stable triple-strand intermediate in the present invention. Triple-strand structures occur rarely, if at all, under physiological conditions and are limited to very unusual base sequence with long runs of purines and pyrimidines. By contrast, dsRNA-mediated inhibition occurs efficiently under physiological conditions, and occurs with a wide variety of inhibitory and target nucleotide sequences. The present invention has inhibited expression of 18 different genes, providing phenocopies of null mutations in these genes

Printed: 22-04-2005 TIPAT 99910592

of known function. These distinctions make it unlikely that dsRNA-mediated inhibition *in C. elegans* is mediated by a triple-strand structure.

Distinction between Present Invention and Co-Suppression Approaches

The transgene-mediated genetic interference phenomenon called co-suppression may include a wide variety of different processes. From the viewpoint of application to other types of organisms, the co-suppression phenomenon in plants is difficult to extend. A confounding aspect in creating a general technique based on co-suppression is that some transgenes in plants lead to suppression of the endogenous locus and some do not. Results in *C. elegans* and *Drosophila* indicate that certain transgenes can cause interference (i.e., a quantitative decrease in the activity of the corresponding endogenous locus) but that most transgenes do not produce such an effect. The lack of a predictable effect in plants, nematodes, and insects greatly limits the usefulness of simply adding transgenes to the genome to interfere with gene expression. Viral-mediated co-suppression in plants appears to be quite effective, but has a number of drawbacks. First, it is not clear what aspects of the viral structure are critical for the observed interference. Extension to another system would require discovery of a virus in that system which would have these properties, and such a library of useful viral agents are not available for many organisms. Second, the use of a replicating virus within an organism to effect genetic changes (e.g., long- or short-term gene therapy) requires considerably more monitoring and oversight for deleterious effects than the use of a defined and non-replicating nucleic acid as in the present invention.

The present invention avoids the disadvantages of the previously-described methods for genetic interference. Several advantages of the present invention are discussed below, but numerous others will be apparent to one of ordinary skill in the biotechnology and genetic engineering arts.

SUMMARY OF THE INVENTION

A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character

66 20-04-2005

into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. The process may be practiced *ex vivo* or *in vivo*. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell (i.e., a cellular gene), an endogenous gene (i.e., a cellular gene present in the genome), a transgene (i.e., a gene construct inserted at an ectopic site in the genome of the cell), or a viral gene which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of target gene transcription, accumulation of target mRNA, or translation of target protein.

The RNA may comprise one or more strands of polymerized ribonucleotide; it may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or multiple complementary RNA strands. Fully duplex RNA lacks an unpaired region with single-stranded structure. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, or 1000 copies per cell) of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. RNA containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by alignment algorithms known in

the art (see Gribskov and Devereux, *Sequence Analysis Primer*, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences. Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and target gene is preferred for the identical nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C for 12-16 hr). The length of the identical nucleotide sequences may be at least 25, 50, 100, 200 or 400 nucleotides.

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, fungus or yeast). RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or in *vitro*. For transcription from a transgene *in vivo* or an expression vector, a regulatory region (e.g., promoter, enhancer, silencer) is used to transcribe the RNA strand(s).

The RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, or circulation of an organism. Physical methods of introducing nucleic acids are preferred such as injection into the cell or extracellular injection of a solution containing the RNA.

The advantages of the invention include: the ease of introducing double-stranded RNA into cells, the low concentration of RNA that is used, the stability of double-stranded RNA, and the effectiveness of the inhibition. The ability to use a low concentration of a naturally-occurring nucleic acid avoids several disadvantages of antisense interference. The present invention is not limited to *in vitro* use or to specific sequence compositions, as are techniques based on triple-strand formation. And unlike antisense interference, triple-strand interference, and co-suppression, the present invention does not suffer from being limited to a particular set of target genes, a particular portion of the target gene's nucleotide sequence, or a particular transgene or viral delivery method. The noted disadvantages have been a serious obstacle to designing general

Printed: 22-04-2005 TIPA1 99910592

strategies according to the prior art for inhibiting gene expression of a target gene of interest.

Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; *unc-22* [9], *unc-54* [12], *fem-1* [14], and *hlh-1* [15]). These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for RNAi is designated with the name of the gene followed by a single letter (e.g., *unc22C*). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.

Figures 2 A-H show analysis of inhibitory RNA effects in individual cells. These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene expression. The micrographs show progeny of injected animals. Panels A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (ds-*unc22A*). These GFP patterns appear identical to the parent strain, with prominent fluorescence in nuclei (the nuclear localized GFP-

69 20-04-2005

LacZ) and mitochondria (the mitochondrially targeted GFP). Panels D-F show progeny of animals injected with ds-*gfpG*. Observable GFP fluorescence is completely absent in over 95% of cells. Only a single active cell is seen in the larva in panel D, while the adult animal in panel E shows staining in none of the striated body wall muscles. Inhibition is not effective in all tissues: the entire vulval musculature expresses active GFP in the adult animal shown in panel E. Panel F shows two rare GFP positive cells in an adult. Both cells express both nuclear-targeted GFP-LacZ and mitochondrial GFP. Panels G-I demonstrate specificity: animals are injected with ds-*lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. In the animals derived from this injection, mitochondrial-targeted GFP appears unaffected while the nuclear-targeted GFP-LacZ is absent from almost all cells (e.g., larva in panel G). Panel H shows a typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars are 20 μm.

Figures 3 A-D show effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA. Micrographs show *in situ* hybridization to embryos. The 1262 nt *mex-3* cDNA clone[20] was divided into two segments, *mex-3*A and *mex-3*B with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. *mex-3*B antisense or dsRNA was injected into the gonads of adult animals, which were fed for 24 hours before fixation and *in situ* hybridization (see reference 5). The *mex-3*B dsRNA produced 100% embryonic arrest, while >90% of embryos from the antisense injections hatched. Antisense probes corresponding to *mex-3*A were used to assay distribution of the endogenous *mex-3* mRNA (dark stain). Four-cell stage embryos are shown; similar results were observed from the 1 to 8 cell stage and in the germline of injected adults. Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous *mex-3* RNA[20]). Panel C: Embryo from a parent injected with purified *mex-3*B antisense RNA. These embryos and the parent animals retain the *mex-3* mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with dsRNA corresponding to

*mex-3*B; no *mex-3* RNA was detected. The scale is such that each embryo is approximately 50 μm in length.

Figure 4 shows inhibitory activity of *unc-22A* as a function of structure and concentration. Purified antisense and sense RNA from *unc22A* were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (*gfpG*). Injected animals were transferred to fresh culture plates 6, 15, 27, 41 and 56 hours after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included in the graph. Bottom-left: diagram showing genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids[8,3].

DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention provides a method of sequence-specific inhibition of gene expression with double-stranded RNA.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. Sequence specificity refers to the ability to inhibit the target gene without manifest effects on other genes of a cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, Northern hybridization, reverse transcription, antibody binding, enzyme linked immunosorbent assay, Western blotting, radioimmunoassay, and fluorescence activated cell analysis. For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include alkaline phosphatase, beta galactosidase (LacZ), chloramphenicol acetyltransferase, luciferase, green fluorescent protein (GFP), beta glucoronidase, or derivatives thereof. Depending on the assay, quantitation of the amount of gene

expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99%.

The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, fungus or yeast. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Plants include corn, wheat, tobacco, arabidopsis, soybean, cauliflower, potato, pine, rubber, and oak. Examples of vertebrate animals include fish, mammal, cow, goat, pig, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, Drosophila and other insects.

That cell may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

RNA may be chemically synthesized by manual or automated reactions. The RNA may be synthesized by RNA polymerase of the cell or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and construction of an expression vector are known in the art (see Goeddel, *Gene Expression Technology*, Academic Press, 1990; Kriegler, *Gene Transfer and Expression*, Stockton Press, 1990; Murray, *Gene Transfer and Expression Protocols*, Humana Press, 1991; WO 97/32016; and references cited therein). If synthesized chemically or by *in vitro* enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Preferably, the RNA is used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing.

The RNA may be introduced directly into the cell (i.e., intracellularly) or extracellularly. Examples of extracellular spaces into which the RNA may be introduced include a cavity, interstitial space, or the circulation of an organism. We disclose herein that in *C. elegans*, double-stranded RNA introduced outside the cell inhibits gene expression. Vascular or extravascular circulation, the blood or lymph system, the phloem, and cerebrospinal fluid are sites where the RNA may be introduced.

Physical methods of introducing nucleic acids are preferred such as for example, injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes with the RNA. A viral vector packaged into a viral particle would accomplish both efficient introduction of an expression vector into the cell and transcription of RNA encoded by the expression vector.

The present invention may be used to introduce RNA into a cancerous cell or tumor and thereby inhibit gene expression of a gene required for maintenance of the phenotype. To prevent a disease or other pathology, a target gene may be selected which is required for initiation of the disease or pathology.

The present invention could be used for treatment or development of treatments for cancers of any type, including solid tumors and leukemias, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (e.g., B cell, mixed cell, null cell, T cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast cell, and myeloid), histiocytosis malignant, Hodgkin disease, immunoproliferative small, non-Hodgkin lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, cranio-

pharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adenocarcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, Leydig cell tumor, papilloma, Sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (e.g., Ewing, experimental, Kaposi, and mast cell), neoplasms (e.g., bone, breast, digestive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia, and for treatment of other conditions in which cells have become immortalized.

The operation of the present invention is shown in the model organism *Caenorhabditis elegans*. Introduction of RNA into cells can be used in certain biological systems to interfere with function of an endogenous gene[1,2]. Many such effects are believed to result from a simple antisense mechanism dependent on hybridization between injected single-stranded RNA and endogenous transcripts. In other cases, a more complex mechanism has been suggested. For instance, an RNA interference (RNAi) phenomenon in the nematode *Caenorhabditis elegans* has been used in a variety of studies to manipulate gene expression[3,4].

Despite the usefulness of RNAi in *C. elegans*, many features have been difficult to explain. In particular, the lack of a clear understanding of the critical requirements for

interfering RNA led to a sporadic record of failure and partial success in attempts to extend RNAi beyond the earliest stages following injection. A statement frequently made in the literature was that sense and antisense RNA preparations are each sufficient to cause interference[3,4]. The only precedent for such a situation was in plants where the process of co-suppression had a similar history of usefulness in certain cases, failure in others, and no ability to design interference protocols with a high chance of success. Working with *C. elegans*, we discovered an RNA structure that would give effective and uniform genetic inhibition. The prior art did not teach or suggest that RNA structure was a critical feature for inhibition of gene expression. Indeed the ability of sense and antisense preparations to produce interference[3,4] had been taken as an indication that RNA structure was not a critical factor. Instead, the extensive plant literature and much of the ongoing research in *C. elegans* was focused on the possibility that detailed features of the target gene sequence or its chromosomal locale was the critical feature for interfering with gene expression.

We carefully purified sense or antisense RNA for *unc-22* (see Figure 4) and tested each for interference. It was found that the purified sense and antisense RNAs had only marginal interfering activity. This was unexpected because many techniques in molecular biology are based on the assumption that RNA produced with specific *in vitro* promoters (e.g., T3 or T7 RNA polymerase), or with characterized promoters *in vivo*, is produced predominantly from a single strand. We carried out the purification below to investigate whether a small fraction of the RNA had an unusual structure and that this subpopulation was responsible for the observed genetic inhibition. The unexpected observation that purification greatly diminished the effectiveness of either sense or antisense RNA preparations led to our discovery that RNA structure was responsible for inhibitory activity. We disclose that the non-purified RNA populations that were effective in inhibition assays herein include some molecules with double-stranded character. To rigorously test whether double-stranded character might contribute to genetic inhibition, we carried out additional purification of single-stranded RNAs and compared inhibitory activities of individual strands with that of the double-stranded hybrid.

Printed: 22-04-2005 IPAT 99910592

To The European Patent Office

15 April 2005

Application No. 99910592.7
COMMONWEALTH SCIENTIFIC AND
INDUSTRIAL RESEARCH ORGANISATION

These comments are filed as third party observations under Article 115 EPC.

The Supplementary European Search Report recently issued in connection with the above-mentioned application. However, a number of relevant documents were not cited in that Supplementary Search Report or in the International Search Report that previously issued from the Australian Patent Office. We therefore wish to bring the Examiner's attention to a number of further documents which are relevant to the patentability of the present case.

In particular, the following documents are citable against the above mentioned application under Article 54(3) EPC:

- WO 9932619 (Carnegie Institution of Washington and The University of Massachusetts);
- WO 9949029 (AG-Gene Australia Limited and State of Queensland through its Department of Primary Industries); and
- WO 9853083 (Syngenta Limited).

Each of these PCT applications has been brought into the European regional phase, and a copy of each is enclosed. Their priority documents are also enclosed.

We would also like to inform the Examiner of the following documents, which are citable against the above-mentioned application under Article 54(2) and Article 56 EPC:

- Dorer, D and Henikoff, S, *Cell* 77(7): 993-1002 (1994);
- Sijen, T *et al.*, *The Plant Cell* 8: 2277-2294 (1996);

EPO - DG 1

20. 04. 2005

(93)

20-04-2005

- Jorgensen, R *et al.*, *Mol. Gen. Genet.* 207: 471-477 (1987);
- WO 97/01952 (DNA Plant Technology Corporation);
- WO 94/01550 (Hybridon Inc.);
- Kuipers, A *et al.*, *Mol. Gen. Genet.* 246: 745-755 (1995);
- Hergersberg M, Inaugural-Dissertation, Universität Köln (1998);
- "Safety Assessment of Genetically Engineered Fruits and Vegetables - A Case Study of the FlavrSavr™ Tomato", CRC Press (1992); and
- Van Blokland, R *et al.*, Post-transcriptional suppression of chalcone synthase genes in *Petunia hybrida* and the accumulation of unspliced pre-mRNAs, Mechanisms and Applications of Gene Silencing, 57-69, Grierson *et al.* (Eds), Nottingham University Press (1996).

A copy of each of these documents is also enclosed.

Further, a document by:

- Fire, A *et al.* (*Nature* 391: 806-811 (1998))

that was cited in the International Search Report in category A is in fact pertinent to the inventive step of the above-mentioned application and is more relevant than its categorization in the International Search Report indicates.

We would also like the Examiner to be aware of the fact that nine oppositions have been filed against European patent EP-B1-0983370 resulting from WO 9853083 (Syngenta Limited), mentioned above. A consultation of the EPO's file for that patent reveals a large number of prior art documents that are also potentially relevant to the present case.

Additionally, eight oppositions have been filed against a patent by Ribopharma AG (EP-B1-1144623) that is also in this technical field. The EPO's file for that case also contains prior art that is potentially relevant to the present case.

Lastly, relevant prior art is also contained in the EPO's files relating to examination of the three Article 54(3) documents mentioned above.

Printed: 22-04-2005

TIPAT

99910592

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/11, 15/63, 15/82

A1

(11) International Publication Number: WO 99/32619

(43) International Publication Date: 1 July 1999 (01.07.99)

(21) International Application Number: PCT/US98/27233

(22) International Filing Date: 21 December 1998 (21.12.98)

(30) Priority Data:

60/068,562	23 December 1997 (23.12.97)	US
09/215,257	18 December 1998 (18.12.98)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	60/068,562 (CON)
Filed on	23 December 1997 (23.12.97)
US	09/215,257 (CON)
Filed on	18 December 1998 (18.12.98)

(71) Applicants *(for all designated States except US)*: THE CARNEGIE INSTITUTE OF WASHINGTON [US/US]; 1530 P Street, N.W., Washington, DC 20005 (US). THE UNIVERSITY OF MASSACHUSETTS [US/US]; One Beacon Street, Boston, MA 02108 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: FIRE, Andrew [US/US]; 2320 Bright Leaf Way, Baltimore, MD 21210 (US). XU, Siqun [CN/US]; 1755 Warminton Court, Ballwin, MO 63021 (US). MONTGOMERY, Mary, K. [US/US]; 233 Macalester Street, St. Paul, MN 55105 (US). KOSTAS, Stephen, A. [US/US]; 126 East Melrose Avenue, Baltimore, MD 21212 (US). TIMMONS, Lisa [US/US]; 2408 Brambleton Road, Baltimore, MD 21209 (US). TABARA, Hiroaki [JP/US]; Apartment #1, 145 Orient Street, Worcester, MA 01604 (US). DRIVER, Samuel, E. [US/US]; Apartment #4, 1714 Commonwealth Avenue, Brighton, MA 02135 (US). MELLO, Craig, C. [US/US]; 19 Ryan Road, Shrewsbury, MA 01545 (US).

(74) Agents: KOKULIS, Paul, N. et al.; Pillsbury Madison & Sutro LLP, 1100 New York Avenue, N.W., Washington, DC 20005 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GENETIC INHIBITION BY DOUBLE-STRANDED RNA

(57) Abstract

A process is provided of introducing an RNA into a living cell to inhibit gene expression of a target gene in that cell. The process may be practiced *ex vivo* or *in vivo*. The RNA has a region with double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequences of the duplex region of the RNA and of a portion of the target gene are identical. The present invention is distinguished from prior art interference in gene expression by antisense or triple-strand methods.

EPO - DG 1

20. 04. 2005

93

20-04-2005

Filed: 22-04-2005

99910592

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

20-04-2005

Printed: 22-04-2005 EPAT 99/0592

GENETIC INHIBITION BY DOUBLE-STRANDED RNA

GOVERNMENT RIGHTS

This invention was made with U.S. government support under grant numbers GM-37706, GM-17164, HD-33769 and GM-07231 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to gene-specific inhibition of gene expression by double-stranded ribonucleic acid (dsRNA).

2. Description of the Related Art

Targeted inhibition of gene expression has been a long-felt need in biotechnology and genetic engineering. Although a major investment of effort has been made to achieve this goal, a more comprehensive solution to this problem was still needed.

Classical genetic techniques have been used to isolate mutant organisms with reduced expression of selected genes. Although valuable, such techniques require laborious mutagenesis and screening programs, are limited to organisms in which genetic manipulation is well established (e.g., the existence of selectable markers, the ability to control genetic segregation and sexual reproduction), and are limited to applications in which a large number of cells or organisms can be sacrificed to isolate the desired mutation. Even under these circumstances, classical genetic techniques can fail to produce mutations in specific target genes of interest, particularly when complex genetic pathways are involved. Many applications of molecular genetics require the ability to go beyond classical genetic screening techniques and efficiently produce a *directed* change in gene expression in a specified group of cells or organisms. Some such applications are knowledge-based projects in which it is of importance to understand what effects the loss of a specific gene product (or products) will have on the behavior of the cell or organism. Other applications are engineering based, for example: cases in which is important to

20-04-2005

Printed 22-04-2005 TIPAT 99910592

produce a population of cells or organisms in which a specific gene product (or products) has been reduced or removed. A further class of applications is therapeutically based in which it would be valuable for a functioning organism (e.g., a human) to reduce or remove the amount of a specified gene product (or products). Another class of applications provides a disease model in which a physiological function in a living organism is genetically manipulated to reduce or remove a specific gene product (or products) without making a permanent change in the organism's genome.

In the last few years, advances in nucleic acid chemistry and gene transfer have inspired new approaches to engineer specific interference with gene expression. These approaches are described below.

Use of Antisense Nucleic Acids to Engineer Interference

Antisense technology has been the most commonly described approach in protocols to achieve gene-specific interference. For antisense strategies, stochiometric amounts of single-stranded nucleic acid complementary to the messenger RNA for the gene of interest are introduced into the cell. Some difficulties with antisense-based approaches relate to delivery, stability, and dose requirements. In general, cells do not have an uptake mechanism for single-stranded nucleic acids, hence uptake of unmodified single-stranded material is extremely inefficient. While waiting for uptake into cells, the single-stranded material is subject to degradation. Because antisense interference requires that the interfering material accumulate at a relatively high concentration (at or above the concentration of endogenous mRNA), the amount required to be delivered is a major constraint on efficacy. As a consequence, much of the effort in developing antisense technology has been focused on the production of modified nucleic acids that are both stable to nuclease digestion and able to diffuse readily into cells. The use of antisense interference for gene therapy or other whole-organism applications has been limited by the large amounts of oligonucleotide that need to be synthesized from non-natural analogs, the cost of such synthesis, and the difficulty even with high doses of maintaining a sufficiently concentrated and uniform pool of interfering material in each cell.

20-04-2005

Printed: 22-04-2005 TIPA 999 0592

Triple-Helix Approaches to Engineer Interference

A second, proposed method for engineered interference is based on a triple helical nucleic acid structure. This approach relies on the rare ability of certain nucleic acid populations to adopt a triple-stranded structure. Under physiological conditions, nucleic acids are virtually all single- or double-stranded, and rarely if ever form triple-stranded structures. It has been known for some time, however, that certain simple purine- or pyrimidine-rich sequences could form a triple-stranded molecule *in vitro* under extreme conditions of pH (i.e., in a test tube). Such structures are generally very transient under physiological conditions, so that simple delivery of unmodified nucleic acids designed to produce triple-strand structures does not yield interference. As with antisense, development of triple-strand technology for use *in vivo* has focused on the development of modified nucleic acids that would be more stable and more readily absorbed by cells *in vivo*. An additional goal in developing this technology has been to produce modified nucleic acids for which the formation of triple-stranded material proceeds effectively at physiological pH.

Co-Suppression Phenomena and Their Use in Genetic Engineering

A third approach to gene-specific interference is a set of operational procedures grouped under the name "co-suppression". This approach was first described in plants and refers to the ability of transgenes to cause silencing of an unlinked but homologous gene. More recently, phenomena similar to co-suppression have been reported in two animals: *C. elegans* and *Drosophila*. Co-suppression was first observed by accident, with reports coming from groups using transgenes in attempts to achieve over-expression of a potentially useful locus. In some cases the over-expression was successful while, in many others, the result was opposite from that expected. In those cases, the transgenic plants actually showed less expression of the endogenous gene. Several mechanisms have so far been proposed for transgene-mediated co-suppression in plants; all of these mechanistic proposals remain hypothetical, and no definitive mechanistic description of the process has been presented. The models that have been proposed to explain co-suppression can be placed in two different categories. In one set of proposals, a direct physical interaction at the DNA- or chromatin-level between two different chromosomal sites has been

20-04-2005

hypothesized to occur; an as-yet-unidentified mechanism would then lead to *de novo* methylation and subsequent suppression of gene expression. Alternatively, some have postulated an RNA intermediate, synthesized at the transgene locus, which might then act to produce interference with the endogenous gene. The characteristics of the interfering RNA, as well as the nature of the interference process, have not been determined. Recently, a set of experiments with RNA viruses have provided some support for the possibility of RNA intermediates in the interference process. In these experiments, a replicating RNA virus is modified to include a segment from a gene of interest. This modified virus is then tested for its ability to interfere with expression of the endogenous gene. Initial results with this technique have been encouraging, however, the properties of the viral RNA that are responsible for interference effects have not been determined and, in any case, would be limited to plants which are hosts of the plant virus.

Distinction between the Present Invention and Antisense Approaches

The present invention differs from antisense-mediated interference in both approach and effectiveness. Antisense-mediated genetic interference methods have a major challenge: delivery to the cell interior of specific single-stranded nucleic acid molecules at a concentration that is equal to or greater than the concentration of endogenous mRNA. Double-stranded RNA-mediated inhibition has advantages both in the stability of the material to be delivered and the concentration required for effective inhibition. Below, we disclose that in the model organism *C. elegans*, the present invention is at least 100-fold more effective than an equivalent antisense approach (i.e., dsRNA is at least 100-fold more effective than the injection of purified antisense RNA in reducing gene expression). These comparisons also demonstrate that inhibition by double-stranded RNA must occur by a mechanism distinct from antisense interference.

Distinction between the Present Invention and Triple-Helix Approaches

The limited data on triple strand formation argues against the involvement of a stable triple-strand intermediate in the present invention. Triple-strand structures occur rarely, if at all, under physiological conditions and are limited to very unusual base sequence with long runs of purines and pyrimidines. By contrast, dsRNA-mediated

Printed: 22-04-2005 TIPAT 99910592

inhibition occurs efficiently under physiological conditions, and occurs with a wide variety of inhibitory and target nucleotide sequences. The present invention has been used to inhibit expression of 18 different genes, providing phenocopies of null mutations in these genes of known function. The extreme environmental and sequence constraints on triple-helix formation make it unlikely that dsRNA-mediated inhibition *in C. elegans* is mediated by a triple-strand structure.

Distinction between Present Invention and Co-Suppression Approaches

The transgene-mediated genetic interference phenomenon called co-suppression may include a wide variety of different processes. From the viewpoint of application to other types of organisms, the co-suppression phenomenon in plants is difficult to extend. A confounding aspect in creating a general technique based on co-suppression is that some transgenes in plants lead to suppression of the endogenous locus and some do not. Results in *C. elegans* and *Drosophila* indicate that certain transgenes can cause interference (i.e., a quantitative decrease in the activity of the corresponding endogenous locus) but that most transgenes do not produce such an effect. The lack of a predictable effect in plants, nematodes, and insects greatly limits the usefulness of simply adding transgenes to the genome to interfere with gene expression. Viral-mediated co-suppression in plants appears to be quite effective, but has a number of drawbacks. First, it is not clear what aspects of the viral structure are critical for the observed interference. Extension to another system would require discovery of a virus in that system which would have these properties, and such a library of useful viral agents are not available for many organisms. Second, the use of a replicating virus within an organism to effect genetic changes (e.g., long- or short-term gene therapy) requires considerably more monitoring and oversight for deleterious effects than the use of a defined nucleic acid as in the present invention.

The present invention avoids the disadvantages of the previously-described methods for genetic interference. Several advantages of the present invention are discussed below, but numerous others will be apparent to one of ordinary skill in the biotechnology and genetic engineering arts.

20-04-2005

Printed: 22-04-2005 TIPAT 99910592

SUMMARY OF THE INVENTION

A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

The RNA may comprise one or more strands of polymerized ribonucleotide; it may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. RNA containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be

19 20-04-2005

defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or in *vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region may be used to transcribe the RNA strand (or strands).

The RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

The advantages of the present invention include: the ease of introducing double-stranded RNA into cells, the low concentration of RNA which can be used, the stability of double-stranded RNA, and the effectiveness of the inhibition. The ability to use a low concentration of a naturally-occurring nucleic acid avoids several disadvantages of antisense interference. This invention is not limited to *in vitro* use or to specific sequence compositions, as are techniques based on triple-strand formation. And unlike antisense interference, triple-strand interference, and co-suppression, this invention does not suffer from being limited to a particular set of target genes, a particular portion of the target gene's nucleotide sequence, or a particular transgene or viral delivery method. These concerns have been a serious obstacle to designing general strategies according to the prior art for inhibiting gene expression of a target gene of interest.

Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease

Printed: 22-04-2005 TIPA 99910592

models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; *unc-22* [9], *unc-54* [12], *fem-1* [14], and *hlh-1* [15]).

Figures 2 A-I show analysis of inhibitory RNA effects in individual cells. These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins, nuclear GFP-LacZ and mitochondrial GFP. The micrographs show progeny of injected animals visualized by a fluorescence microscope. Panels A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (ds-*unc22A*). Panels D-E show progeny of animals injected with ds-*gfpG*. Panels G-I demonstrate specificity. Animals are injected with ds-*lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. Panel H shows a typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars are 20 μm.

Figures 3 A-D show effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA. Micrographs show *in situ* hybridization to embryos (dark stain). Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous *mex-3* RNA[20]). Panel C: Embryo from a parent injected with purified *mex-3*B antisense RNA. These embryos and the parent animals retain the *mex-3* mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with dsRNA corresponding to *mex-3*B; no *mex-3* RNA was detected. Scale: each embryo is approximately 50 μm in length.

Figure 4 shows inhibitory activity of *unc-22A* as a function of structure and concentration. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included. Progeny cohort groups are labeled 1 for 0-6 hours, 2 for 6-15

12 20-04-2005

hours, 3 for 15-27 hours, 4 for 27-41 hours, and 5 for 41-56 hours The bottom-left diagram shows genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids[8,3].

Figures 5 A-C show examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. Panel A: General strategy for production of dsRNA by cloning a segment of interest between flanking copies of the bacteriophage T7 promoter and transcribing both strands of the segment by transfecting a bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter. Panel B: A GFP-expressing *C. elegans* strain, PD4251 (see Figure 2), fed on a native bacterial host. Panel C: PD4251 animals reared on a diet of bacteria expressing dsRNA corresponding to the coding region for *gfp*.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing sequence-specific inhibition of gene expression by introducing double-stranded RNA (dsRNA). A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell. Inhibition is sequence-specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell (i.e., a cellular gene), an endogenous gene (i.e., a cellular gene present in the genome), a transgene (i.e., a gene construct inserted at an ectopic site in the genome of the cell), or a gene from a pathogen which is capable of infecting an organism from which the cell is derived. Depending on the particular target gene and the dose of double stranded RNA material delivered, this process may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The

Printed: 22-04-2005 EPA1 99910592

consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

The RNA may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored

14 20-04-2005

to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

RNA containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, *Sequence Analysis Primer*, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 25. 50, 100, 200, 300 or 400 bases.

As disclosed herein, 100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of

Printed: 22-04-2005 IPA 99910592

being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis; field crops (e.g., alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative generae of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tfhchonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphalenchus, Criconemella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, and Xiphinema). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

16 20-04-2005

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or in *vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art[32, 33, 34] (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by *in vitro* enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, intro-

duced orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. For example, the RNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. We disclose herein that in *C. elegans*, double-stranded RNA introduced outside the cell inhibits gene expression. Vascular or extravascular circulation, the blood or lymph system, the phloem, the roots, and the cerebrospinal fluid are sites where the RNA may be introduced. A transgenic organism that expresses RNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.

Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

The present invention may be used to introduce RNA into a cell for the treatment or prevention of disease. For example, dsRNA may be introduced into a cancerous cell or tumor and thereby inhibit gene expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene may be selected which is required for initiation or maintenance of the disease/pathology.

Treatment would include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

A gene derived from any pathogen may be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. The inhibitory RNA could be introduced in cells *in vitro* or *ex vivo* and then subsequently placed into an animal to affect therapy, or directly treated by *in vivo* administration. A method of gene therapy can be envisioned. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, can be envisioned.

The present invention could be used for treatment or development of treatments for cancers of any type, including solid tumors and leukemias, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (e.g., B cell, mixed cell, null cell, T cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast cell, and myeloid), histiocytosis malignant, Hodgkin disease, immunoproliferative small, non-Hodgkin lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, cranio-pharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adeno-

carcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, Leydig cell tumor, papilloma, Sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (e.g., Ewing, experimental, Kaposi, and mast cell), neoplasms (e.g., bone, breast, digestive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia, and for treatment of other conditions in which cells have become immortalized or transformed. The invention could be used in combination with other treatment modalities, such as chemotherapy, cryotherapy, hyperthermia, radiation therapy, and the like.

As disclosed herein, the present invention may is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, TP53, and WT1); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases,

Printed: 22-04-2005 TIPAT 999 0592

amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

The present invention could comprise a method for producing plants with reduced susceptibility to climatic injury, susceptibility to insect damage, susceptibility to infection by a pathogen, or altered fruit ripening characteristics. The targeted gene may be an enzyme, a plant structural protein, a gene involved in pathogenesis, or an enzyme that is involved in the production of a non-proteinaceous part of the plant (i.e., a carbohydrate or lipid). If an expression construct is used to transcribe the RNA in a plant, transcription by a wound- or stress-inducible; tissue-specific (e.g., fruit, seed, anther, flower, leaf, root); or otherwise regulatable (e.g., infection, light, temperature, chemical) promoter may be used. By inhibiting enzymes at one or more points in a metabolic pathway or genes involved in pathogenesis, the effect may be enhanced: each activity will be affected and the effects may be magnified by targeting multiple different components. Metabolism may also be manipulated by inhibiting feedback control in the pathway or production of unwanted metabolic byproducts.

The present invention may be used to reduce crop destruction by other plant pathogens such as arachnids, insects, nematodes, protozoans, bacteria, or fungi. Some such plants and their pathogens are listed in *Index of Plant Diseases in the United States* (U.S. Dept. of Agriculture Handbook No. 165, 1960); *Distribution of Plant-Parasitic Nematode Species in North America* (Society of Nematologists, 1985); and *Fungi on Plants and Plant Products in the United States* (American Phytopathological Society, 1989). Insects with reduced ability to damage crops or improved ability to prevent other destructive insects from damaging crops may be produced. Furthermore, some nematodes are vectors of plant pathogens, and may be attacked by other beneficial nematodes which have no effect on plants. Inhibition of target gene activity could be used to delay or

20-04-2005

prevent entry into a particular developmental step (e.g., metamorphosis), if plant disease was associated with a particular stage of the pathogen's life cycle. Interactions between pathogens may also be modified by the invention to limit crop damage. For example, the ability of beneficial nematodes to attack their harmful prey may be enhanced by inhibition of behavior-controlling nematode genes according to the invention.

Although pathogens cause disease, some of the microbes interact with their plant host in a beneficial manner. For example, some bacteria are involved in symbiotic relationships that fix nitrogen and some fungi produce phytohormones. Such beneficial interactions may be promoted by using the present invention to inhibit target gene activity in the plant and/or the microbe.

Another utility of the present invention could be a method of identifying gene function in an organism comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceutics, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for the yeast, *D. melanogaster*, and *C. elegans* genomes, can be coupled with the invention to determine gene function in an organism (e.g., nematode). The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which RNA can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell/organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96-well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process. Solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The amplified RNA can be fed directly to, injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by *in vivo* or *in vitro* transcription from an expression construct used to produce the library. The construct can be replicated as individual clones of the library and transcribed to produce the RNA; each clone can then be fed to, or injected into, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example: arabidopsis, bacteria, drosophila, fungi, nematodes, viruses, zebrafish, and tissue culture cells derived from mammals.

A nematode or other organism that produces a colorimetric, fluorogenic, or luminescent signal in response to a regulated promoter (e.g., transfected with a reporter gene construct) can be assayed in an HTS format to identify DNA-binding proteins that regulate the promoter. In the assay's simplest form, inhibition of a negative regulator results in an increase of the signal and inhibition of a positive regulator results in a decrease of the signal.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the

characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Pesticides may include the RNA molecule itself, an expression construct capable of expressing the RNA, or organisms transfected with the expression construct. The

pesticide of the present invention may serve as an arachnicide, insecticide, nematicide, viricide, bactericide, and/or fungicide. For example, plant parts that are accessible above ground (e.g., flowers, fruits, buds, leaves, seeds, shoots, bark, stems) may be sprayed with pesticide, the soil may be soaked with pesticide to access plant parts growing beneath ground level, or the pest may be contacted with pesticide directly. If pests interact with each other, the RNA may be transmitted between them. Alternatively, if inhibition of the target gene results in a beneficial effect on plant growth or development, the aforementioned RNA, expression construct, or transfected organism may be considered a nutritional agent. In either case, genetic engineering of the plant is not required to achieve the objectives of the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

Used as either an pesticide or nutrient, a formulation of the present invention may be delivered to the end user in dry or liquid form: for example, as a dust, granulate, emulsion, paste, solution, concentrate, suspension, or encapsulation. Instructions for safe and effective use may also be provided with the formulation. The formulation might be used directly, but concentrates would require dilution by mixing with an extender provided by the formulator or the end user. Similarly, an emulsion, paste, or suspension may require the end user to perform certain preparation steps before application. The formulation may include a combination of chemical additives known in the art such as solid carriers, minerals, solvents, dispersants, surfactants, emulsifiers, tackifiers, binders, and other adjuvants. Preservatives and stabilizers may also be added to the formulation to facilitate storage. The crop area or plant may also be treated simultaneously or separately with other pesticides or fertilizers. Methods of application include dusting, scattering or pouring, soaking, spraying, atomizing, and coating. The precise physical form and chemical composition of the formulation, and its method of application, would be chosen to promote the objectives of the invention and in accordance with prevailing circumstances. Expression constructs and transfected hosts capable of replication may also promote the persistence and/or spread of the formulation.

EPO - DG 1 22-04-2005 IPA1 99910592

Description of the dsRNA Inhibition Phenomenon in *C. elegans*

The operation of the present invention was shown in the model genetic organism *Caenorhabditis elegans*.

Introduction of RNA into cells had been seen in certain biological systems to interfere with function of an endogenous gene[1,2]. Many such effects were believed to result from a simple antisense mechanism dependent on hybridization between injected single-stranded RNA and endogenous transcripts. In other cases, a more complex mechanism had been suggested. One instance of an RNA-mediated mechanism was RNA interference (RNAi) phenomenon in the nematode *C. elegans*. RNAi had been used in a variety of studies to manipulate gene expression[3,4].

Despite the usefulness of RNAi in *C. elegans*, many features had been difficult to explain. Also, the lack of a clear understanding of the critical requirements for interfering RNA led to a sporadic record of failure and partial success in attempts to extend RNAi beyond the earliest stages following injection. A statement frequently made in the literature was that sense and antisense RNA preparations are each sufficient to cause interference[3,4]. The only precedent for such a situation was in plants where the process of co-suppression had a similar history of usefulness in certain cases, failure in others, and no ability to design interference protocols with a high chance of success. Working with *C. elegans*, we discovered an RNA structure that would give effective and uniform genetic inhibition. The prior art did not teach or suggest that RNA structure was a critical feature for inhibition of gene expression. Indeed the ability of crude sense and antisense preparations to produce interference[3,4] had been taken as an indication that RNA structure was not a critical factor. Instead, the extensive plant literature and much of the ongoing research in *C. elegans* was focused on the possibility that detailed features of the target gene sequence or its chromosomal locale was the critical feature for interfering with gene expression.

The inventors carefully purified sense or antisense RNA for *unc-22* and tested each for gene-specific inhibition. While the crude sense and antisense preparations had strong interfering activity, it was found that the purified sense and antisense RNAs had only marginal inhibitory activity. This was unexpected because many techniques in molecular biology are based on the assumption that RNA produced with specific *in vitro*

26 20-04-2005

promoters (e.g., T3 or T7 RNA polymerase), or with characterized promoters *in vivo*, is produced predominantly from a single strand. The inventors had carried out purification of these crude preparations to investigate whether a small fraction of the RNA had an unusual structure which might be responsible for the observed genetic inhibition. To rigorously test whether double-stranded character might contribute to genetic inhibition, the inventors carried out additional purification of single-stranded RNAs and compared inhibitory activities of individual strands with that of the double-stranded hybrid.

The following examples are meant to be illustrative of the present invention; however, the practice of the invention is not limited or restricted in any way by them.

Analysis of RNA-Mediated Inhibition of *C. elegans* Genes

The *unc-22* gene was chosen for initial comparisons of activity as a result of previous genetic analysis that yields a semi-quantitative comparison between *unc-22* gene activity and the movement phenotypes of animals[3,8]: decreases in activity produce an increasingly severe twitching phenotype, while complete loss of function results in the additional appearance of muscle structural defects and impaired motility. *unc-22* encodes an abundant but non-essential myofilament protein[7-9]. *unc-22* mRNA is present at several thousand copies per striated muscle cell[3].

Purified antisense and sense RNAs covering a 742 nt segment of *unc-22* had only marginal inhibitory activity, requiring a very high dose of injected RNA for any observable effect (Figure 4). By contrast, a sense+antisense mixture produced a highly effective inhibition of endogenous gene activity (Figure 4). The mixture was at least two orders of magnitude more effective than either single strand in inhibiting gene expression. The lowest dose of the sense+antisense mixture tested, approximately 60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. *unc-22* expression begins in embryos with approximately 500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent inhibitory activity of the sense+antisense mixture could reflect formation of double-stranded RNA (dsRNA), or conceivably some alternate synergy between the strands. Electrophoretic analysis indicated that the injected material was predomi-

nantly double stranded. The dsRNA was gel purified from the annealed mixture and found to retain potent inhibitory activity. Although annealing prior to injection was compatible with inhibition, it was not necessary. Mixing of sense and antisense RNAs in low salt (under conditions of minimal dsRNA formation), or rapid sequential injection of sense and antisense strands, were sufficient to allow complete inhibition. A long interval (>1 hour) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in inhibitory activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the complementary strand.

An issue of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic response mechanism[10]. Conceivably, the inventive sense+antisense synergy could reflect a non-specific potentiation of antisense effects by such a panic mechanism. This was not found to be the case: co-injection of dsRNA segments unrelated to *unc-22* did not potentiate the ability of *unc-22* single strands to mediate inhibition. Also investigated was whether double-stranded structure could potentiate inhibitory activity when placed in *cis* to a single-stranded segment. No such potentiation was seen; unrelated double-stranded sequences located 5' or 3' of a single-stranded *unc-22* segment did not stimulate inhibition. Thus potentiation of gene-specific inhibition was observed only when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by *unc-22* dsRNA was specific. Progeny of injected animals exhibited behavior indistinguishable from characteristic *unc-22* loss of function mutants. Target-specificity of dsRNA effects using three additional genes with well characterized phenotypes (Figure 1 and Table 1). *unc-54* encodes a body wall muscle myosin heavy chain isoform required for full muscle contraction[7,11,12], *fem-1* encodes an ankyrin-repeat containing protein required in hermaphrodites for sperm production[13,14], and *hlh-1* encodes a *C. elegans* homolog of the myoD family required for proper body shape and motility[15,16]. For each of these genes, injection of dsRNA produced progeny broods exhibiting the known null mutant phenotype, while the purified single strands produced no significant reduction in gene expression. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from inhibition of the corresponding gene. The exception (segment *unc54C*, which led to an embryonic and

larval arrest phenotype not seen with *unc-54* null mutants) was illustrative. This segment covers the highly conserved myosin motor domain, and might have been expected to inhibit the activity of other highly related myosin heavy chain genes[17]. This interpretation would support uses of the present invention in which nucleotide sequence comparison of dsRNA and target gene show less than 100% identity. The *unc54C* segment has been unique in our overall experience to date: effects of 18 other dsRNA segments have all been limited to those expected from characterized null mutants.

The strong phenotypes seen following dsRNA injection are indicative of inhibitory effects occurring in a high fraction of cells. The *unc-54* and *hlh-1* muscle phenotypes, in particular, are known to result from a large number of defective muscle cells[11,16]. To examine inhibitory effects of dsRNA on a cellular level, a transgenic line expressing two different GFP-derived fluorescent reporter proteins in body muscle was used. Injection of dsRNA directed to *gfp* produced dramatic decreases in the fraction of fluorescent cells (Figure 2). Both reporter proteins were absent from the negative cells, while the few positive cells generally expressed both GFP forms.

The pattern of mosaicism observed with *gfp* inhibition was not random. At low doses of dsRNA, the inventors saw frequent inhibition in the embryonically-derived muscle cells present when the animal hatched. The inhibitory effect in these differentiated cells persisted through larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically-derived striated muscles are born during early larval stages and were more resistant to inhibition. These cells have come through additional divisions (13-14 versus 8-9 for embryonic muscles[18,19]). At high concentrations of *gfp* dsRNA, inhibition was noted in virtually all striated bodywall muscles, with occasional single escaping cells including cells born in embryonic or post-embryonic stages. The nonstriated vulval muscles, born during late larval development, appeared resistant to genetic inhibition at all tested concentrations of injected RNA. The latter result is important for evaluating the use of the present invention in other systems. First, it indicates that failure in one set of cells from an organism does not necessarily indicate complete non-applicability of the invention to that organism. Second, it is important to realize that not all tissues in the organism need to be affected for the invention to be used in an organism. This may serve as an advantage in some situations.

Printed 22-04-2005 TIPA 9991/0592

A few observations serve to clarify the nature of possible targets and mechanisms for RNA-mediated genetic inhibition in *C. elegans*:

First, dsRNA segments corresponding to a variety of intron and promoter sequences did not produce detectable inhibition (Table 1). Although consistent with possible inhibition at a post-transcriptional level, these experiments do not rule out inhibition at the level of the gene.

Second, dsRNA injection produced a dramatic decrease in the level of the endogenous mRNA transcript (Figure 3). Here, a *mex-3* transcript that is abundant in the gonad and early embryos[20] was targeted, where straightforward *in situ* hybridization can be performed[5]. No endogenous *mex-3* mRNA was observed in animals injected with a dsRNA segment derived from *mex-3* (Figure 3D), but injection of purified *mex-3* antisense RNA resulted in animals that retained substantial endogenous mRNA levels (Figure 3C).

Third, dsRNA-mediated inhibition showed a surprising ability to cross cellular boundaries. Injection of dsRNA for *unc-22*, *gfp*, or *lacZ* into the body cavity of the head or tail produced a specific and robust inhibition of gene expression in the progeny brood (Table 2). Inhibition was seen in the progeny of both gonad arms, ruling out a transient "nicking" of the gonad in these injections. dsRNA injected into body cavity or gonad of young adults also produced gene-specific inhibition in somatic tissues of the injected animal (Table 2).

Table 3 shows that *C. elegans* can respond in a gene-specific manner to dsRNA encountered in the environment. Bacteria are a natural food source for *C. elegans*. The bacteria are ingested, ground in the animal's pharynx, and the bacterial contents taken up in the gut. The results show that *E. coli* bacteria expressing dsRNAs can confer specific inhibitory effects on *C. elegans* nematode larvae that feed on them.

Three *C. elegans* genes were analyzed. For each gene, corresponding dsRNA was expressed in *E. coli* by inserting a segment of the coding region into a plasmid construct designed for bidirectional transcription by bacteriophage T7 RNA polymerase. The dsRNA segments used for these experiments were the same as those used in previous microinjection experiments (see Figure 1). The effects resulting from feeding these bacteria to *C. elegans* were compared to the effects achieved by microinjecting animals

30 20-04-2005

with dsRNA.

The *C. elegans* gene *unc-22* encodes an abundant muscle filament protein. *unc-22* null mutations produce a characteristic and uniform twitching phenotype in which the animals can sustain only transient muscle contraction. When wild-type animals were fed bacteria expressing a dsRNA segment from *unc-22*, a high fraction (85%) exhibited a weak but still distinct twitching phenotype characteristic of partial loss of function for the *unc-22* gene. The *C. elegans fem-1* gene encodes a late component of the sex determination pathway. Null mutations prevent the production of sperm and lead euploid (XX) animals to develop as females, while wild type XX animals develop as hermaphrodites. When wild-type animals were fed bacteria expressing dsRNA corresponding to *fem-1*, a fraction (43%) exhibit a sperm-less (female) phenotype and were sterile. Finally, the ability to inhibit gene expression of a transgene target was assessed. When animals carrying a *gfp* transgene were fed bacteria expressing dsRNA corresponding to the *gfp* reporter, an obvious decrease in the overall level of GFP fluorescence was observed, again in approximately 12% of the population (see Figure 5, panels B and C).

The effects of these ingested RNAs were specific. Bacteria carrying different dsRNAs from *fem-1* and *gfp* produced no twitching, dsRNAs from *unc-22* and *fem-1* did not reduce *gfp* expression, and dsRNAs from *gfp* and *unc-22* did not produce females. These inhibitory effects were apparently mediated by dsRNA: bacteria expressing only the sense or antisense strand for either *gfp* or *unc-22* caused no evident phenotypic effects on their *C. elegans* predators.

Table 4 shows the effects of bathing *C. elegans* in a solution containing dsRNA. Larvae were bathed for 24 hours in solutions of the indicated dsRNAs (1 mg/ml), then allowed to recover in normal media and allowed to grow under standard conditions for two days. The *unc-22* dsRNA was segment ds-*unc22A* from Figure 1. *pos*-1 and *sqt*-3 dsRNAs were from the full length cDNA clones. *pos-1* encodes an essential maternally provided component required early in embyogenesis. Mutations removing *pos-1* activity have an early embryonic arrest characteristic of *skn*-like mutations[29, 30]. Cloning and activity patterns for *sqt-3* have been described[31]. *C. elegans sqt-3* mutants have mutations in the *col-1* collagen gene[31]. Phenotypes of affected animals are noted. Incidences of

Printed: 22-04-2005 IPEA/US 99910592

clear phenotypic effects in these experiments were 5-10% for *unc-22*, 50% for *pos-1*, and 5% for *sqt-3*. These are frequencies of unambiguous phenocopies; other treated animals may have had marginal defects corresponding to the target gene that were not observable. Each treatment was fully gene-specific in that *unc-22* dsRNA produced only Unc-22 phenotypes, *pos-1* dsRNA produced only Pos-1 phenotypes, and *sqt-3* dsRNA produced only Sqt-3 phenotypes.

Some of the results described herein were published after the filing of our provisional application. Those publications and a review can be cited as Fire, A., et al. Nature, 391, 806-811, 1998; Timmons, L. & Fire, A. Nature, 395, 854, 1998; and Montgomery, M.K. & Fire, A. Trends in Genetics, 14, 255-258, 1998.

The effects described herein significantly augment available tools for studying gene function in *C. elegans* and other organisms. In particular, functional analysis should now be possible for a large number of interesting coding regions[21] for which no specific function have been defined. Several of these observations show the properties of dsRNA that may affect the design of processes for inhibition of gene expression. For example, one case was observed in which a nucleotide sequence shared between several myosin genes may inhibit gene expression of several members of a related gene family.

Methods of RNA Synthesis and Microinjection

RNA was synthesized from phagemid clones with T3 and T7 RNA polymerase[6], followed by template removal with two sequential DNase treatments. In cases where sense, antisense, and mixed RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original "sense" and "antisense" preparations. Nonetheless, RNA species accounting for less than 10% of purified RNA preparations would not have been observed. Without gel purification, the "sense" and "antisense" preparations produced significant inhibition. This inhibitory activity was reduced or eliminated upon gel purification. By contrast, sense+antisense mixtures of gel purified and non-gel-purified RNA preparations produced identical effects.

Following a short (5 minute) treatment at 68°C to remove secondary structure, sense+antisense annealing was carried out in injection buffer[27] at 37°C for 10-30 minutes.

32 20-04-2005

Printed: 22-04-2005 TIPAT 99910592

Formation of predominantly double stranded material was confirmed by testing migration on a standard (non-denaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for double-stranded RNA of the appropriate length. Co-incubation of the two strands in a low-salt buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of double-stranded RNA *in vitro*. Non-annealed sense+antisense RNAs for *unc22B* and *gfpG* were tested for inhibitory effect and found to be much more active than the individual single strands, but 2-4 fold less active than equivalent pre-annealed preparations.

After pre-annealing of the single strands for *unc22A*, the single electrophoretic species corresponding in size to that expected for dsRNA was purified using two rounds of gel electrophoresis. This material retained a high degree of inhibitory activity.

Except where noted, injection mixes were constructed so animals would receive an average of 0.5×10^6 to 1.0×10^6 molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections were compared with equal masses of RNA (i.e., dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible; however, such variability would not affect any of the conclusions drawn herein.

Methods for Analysis of Phenotypes

Inhibition of endogenous genes was generally assayed in a wild type genetic background (N2). Features analyzed included movement, feeding, hatching, body shape, sexual identity, and fertility. Inhibition with *gfp* [27] and *lacZ* activity was assessed using strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccIs4251) made up of three plasmids: pSAK4 (*myo-3* promoter driving mitochondrially targeted GFP), pSAK2 (*myo-3* promoter driving a nuclear targeted GFP-LacZ fusion), and a *dpy-20* subclone[26] as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing a facile distinction between

20-04-2005

Printed: 22-04-2005 IPA 99910592

cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was performed by inserting the microinjection needle into the gonadal syncitium of adults and expelling 20-100 pl of solution (see Reference 25). Body cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells was another effective means of RNA delivery, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16 hour intervals. This yields a series of semi-synchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short "clearance" interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. After the clearance period, individuals are produced which show the inhibitory phenotype. After injected animals have produced eggs for several days, gonads can in some cases "revert" to produce incompletely affected or phenotypically normal progeny.

Additional Description of the Results

Figure 1 shows genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; sequence references are as follows: *unc-22* [9], *unc-54* [12], *fem-1* [14], and *hlh-1* [15]). These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for inhibitory effects is designated with the name of the gene followed by a single letter (e.g., *unc22C*). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.

The effects of inhibitory RNA were analyzed in individual cells (Figure 2, panels A-H). These experiments were carried out in a reporter strain (called PD4251) expressing

84 20-04-2005

two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene. ds-*unc22A* RNA was injected as a negative control. GFP expression in progeny of these injected animals was not affected. The GFP patterns of these progeny appeared identical to the parent strain, with prominent fluorescence in nuclei (the nuclear localized GFP-LacZ) and mitochondria (the mitochondrially targeted GFP): young larva (Figure 2A), adult (Figure 2B), and adult body wall at high magnification (Figure 2C).

In contrast, the progeny of animals injected with ds-*gfpG* RNA are affected (Figures 2D-F). Observable GFP fluorescence is completely absent in over 95% of the cells. Few active cells were seen in larvae (Figure 2D shows a larva with one active cell; uninjected controls show GFP activity in all 81 body wall muscle cells). Inhibition was not effective in all tissues: the entire vulval musculature expressed active GFP in an adult animal (Figure 2E). Rare GFP positive body wall muscle cells were also seen adult animals (two active cells are shown in Figure 2F). Inhibition was target specific (Figures 2G-I). Animals were injected with ds-*lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. In the animals derived from this injection, mitochondrial-targeted GFP appeared unaffected while the nuclear-targeted GFP-LacZ was absent from almost all cells (larva in Figure 2G). A typical adult lacked nuclear GFP-LacZ in almost all body-wall muscles but retained activity in vulval muscles (Figure 2H). Scale bars in Figure 2 are 20 μm.

The effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA was shown by *in situ* hybridization to embryos (Figure 3, panels A-D). The 1262 nt *mex-3* cDNA clone[20] was divided into two segments, *mex-3*A and *mex-3*B with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. *mex-3*B antisense or dsRNA was injected into the gonads of adult animals, which were maintained under standard culture conditions for 24 hours before fixation and *in situ* hybridization (see Reference 5). The *mex-3*B dsRNA produced 100% embryonic arrest, while >90% of embryos from the antisense injections hatched. Antisense probes corresponding to *mex-3*A were used to

Printed: 22-04-2005 TIPA 99910592

assay distribution of the endogenous *mex-3* mRNA (dark stain). Four-cell stage embryos were assayed; similar results were observed from the 1 to 8 cell stage and in the germline of injected adults. The negative control (the absence of hybridization probe) showed a lack of staining (Figure 3A). Embryos from uninjected parents showed a normal pattern of endogenous *mex-3* RNA (Figure 3B). The observed pattern of *mex-3* RNA was as previously described in Reference 20. Injection of purified *mex-3*B antisense RNA produced at most a modest effect: the resulting embryos retained *mex-3* mRNA, although levels may have been somewhat less than wild type (Figure 3C). In contrast, no *mex-3* RNA was detected in embryos from parents injected with dsRNA corresponding to *mex-3*B (Figure 3D). The scale of Figure 3 is such that each embryo is approximately 50 μm in length.

Gene-specific inhibitory activity by *unc-22A* RNA was measured as a function of RNA structure and concentration (Figure 4). Purified antisense and sense RNA from *unc22A* were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (*gfpG*). Injected animals were transferred to fresh culture plates 6 hours (columns labeled 1), 15 hours (columns labeled 2), 27 hours (columns labeled 3), 41 hours (columns labeled 4), and 56 hours (columns labeled 5) after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included in the graph. The bottom-left diagram shows the genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids[8,3].

Figures 5 A-C show a process and examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. A general strategy for production of dsRNA is to clone segments of interest between flanking copies of the bacteriophage T7 promoter into a bacterial plasmid construct (Figure 5A). A bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter was used as a host. A nuclease-resistant dsRNA was detected in lysates of transfected

36 20-04-2005

Printed: 22-04-2005 TIPA 99940592

bacteria. Comparable inhibition results were obtained with the two bacterial expression systems. A GFP-expressing *C. elegans* strain, PD4251 (see Figure 2), was fed on a native bacterial host. These animals show a uniformly high level of GFP fluorescence in body muscles (Figure 5B). PD4251 animals were also reared on a diet of bacteria expressing dsRNA corresponding to the coding region for *gfp*. Under the conditions of this experiment, 12% of these animals showed dramatic decreases in GFP (Figure 5C). As an alternative strategy, single copies of the T7 promoter were used to drive expression of an inverted-duplication for a segment of the target gene, either *unc-22* or *gfp*. This was comparably effective.

All references (e.g., books, articles, applications, and patents) cited in this specification are indicative of the level of skill in the art and their disclosures are incorporated herein in their entirety.

1. Izant, J. & Weintraub, H. *Cell* 36, 1007-1015 (1984).
2. Nellen, W. & Lichtenstein, C. *TIBS* 18, 419-423 (1993).
3. Fire, A., et al. *Development* 113, 503-514 (1991).
4. Guo, S. & Kemphues, K. *Cell* 81, 611-620 (1995).
5. Seydoux, G. & Fire, A. *Development* 120, 2823-2834 (1994).
6. Ausubel, F., et al. *Current Protocols in Molecular Biology*, John Wiley N.Y. (1990).
7. Brenner, S. *Genetics* 77, 71-94 (1974).
8. Moerman, D. & Baillie, D. *Genetics* 91, 95-104 (1979).
9. Benian, G., et al. *Genetics* 134, 1097-1104 (1993).
10. Proud, C. *TIBS* 20, 241-246 (1995).
11. Epstein H., et al. *J. Mol. Biol.* 90 291-300 (1974).
12. Karn, J., et al. *Proc. Natl. Acad. Sci. (U.S.A.)* 80, 4253-4257 (1983).
13. Doniach, T. & Hodgkin J.A. *Dev. Biol.* 106, 223-235 (1984).
14. Spence, A., et al. *Cell* 60, 981-990 (1990).
15. Krause, M., et al. *Cell* 63, 907-919 (1990).
16. Chen, L., et al. *Development*, 120, 1631-1641 (1994).
17. Dibb, N. J., et al. *J. Mol. Biol.* 205, 603-613 (1989).

37 20-04-2005

Printed: 22-04-2005 TIPA1 99910592

18. Sulston, J., et al. *Dev. Biol.* 100, 64-119 (1983).

19. Sulston, J. & Horvitz, H. *Dev. Biol.* 82, 41-55 (1977).

20. Draper B.W., et al. *Cell* 87, 205-216 (1996).

21. Sulston, J., et al. *Nature* 356, 37-41 (1992).

22. Matzke, M. & Matzke, A. *Plant Physiol.* 107, 679-685 (1995).

23. Ratcliff, F., et al. *Science* 276, 1558-1560 (1997).

24. Latham, K. *Trends in Genetics* 12,134-138 (1996).

25. Mello, C. & Fire, A. *Methods in Cell Biology* 48, 451-482 (1995).

26. Clark, D., et al. *Mol. Gen. Genet.* 247, 367-378 (1995).

27. Chalfie, M., et al. *Science* 263, 802-805 (1994).

28. Studier, F., et al. *Methods in Enzymology* 185, 60-89 (1990).

29. Bowerman, B., et al. *Cell* 68, 1061-1075 (1992).

30. Mello, C.C., et al. *Cell* 70, 163-176 (1992).

31. van der Keyl, H., et al. *Develop. Dynamics* 201, 86-94 (1994).

32. Goeddel, D.V. *Gene Expression Technology*, Academic Press, 1990.

33. Kriegler, M. *Gene Transfer and Expression*, Stockton Press, 1990.

34. Murray, E.J. *Gene Transfer and Expression Protocols*, Humana Press, 1991.

20-04-2005

Printed: 22-04-2005 TIPAT 99910592

Table 1. Effects of sense, antisense, and mixed RNAs on progeny of injected animals.

Gene and Segment		Size	Injected RNA	F1 Phenotype
unc-22			***unc-22* null mutants: strong twitchers**[7,8]	
unc22A[a]	exon 21-22	742	sense	wild type
			antisense	wild type
			sense+antisense	strong twitchers (100%)
unc22B	exon 27	1033	sense	wild type
			antisense	wild type
			sense+antisense	strong twitchers (100%)
unc22C	exon 21-22[b]	785	sense+antisense	strong twitchers (100%)
fem-1			***fem-1* null mutants: female (no sperm)**[13]	
fem1A	exon 10[c]	531	sense	hermaphrodite (98%)
			antisense	hermaphrodite (>98%)
			sense+antisense	female (72%)
fem1B	intron 8	556	sense+antisense	hermaphrodite (>98%)
unc-54			***unc-54* null mutants: paralyzed**[7,11]	
unc54A	exon 6	576	sense	wild type (100%)
			antisense	wild type (100%)
			sense+antisense	paralyzed (100%)[d]
unc54B	exon 6	651	sense	wild type (100%)
			antisense	wild type (100%)
			sense+antisense	paralyzed (100%)[d]
unc54C	exon 1-5	1015	sense+antisense	arrested embryos and larvae (100%)
unc54D	promoter	567	sense+antisense	wild type (100%)
unc54E	intron 1	369	sense+antisense	wild type (100%)
unc54F	intron 3	386	sense+antisense	wild type (100%)

SIP 20-04-2005

Printed: 22-04-2005 IPA1 99910592

Table 1 (continued).

	Gene and Segment		Size	Injected RNA	F1 Phenotype
	hlh-1			***hlh-1* null mutants: lumpy-dumpy larvae**[16]	
	hlh1A	exons 1-6	1033	sense	wild type (<2% lpy-dpy)
				antisense	wild type (<2% lpy-dpy)
				sense+antisense	lpy-dpy larvae (>90%)[e]
	hlh1B	exons 1-2	438	sense+antisense	lpy-dpy larvae (>80%)[e]
	hlh1C	exons 4-6	299	sense+antisense	lpy-dpy larvae (>80%)[e]
	hlh1D	intron 1	697	sense+antisense	wild type (<2% lpy-dpy)
	myo-3 driven GFP transgenes[f]				
	myo-3::NLS::gfp::lacZ			**makes nuclear GFP in body muscle**	
	gfpG	exons 2-5	730	sense	nuclear GFP-LacZ pattern of parent strain
				antisense	nuclear GFP-LacZ pattern of parent strain
				sense+antisense	nuclear GFP-LacZ absent in 98% of cells
	lacZL	exon 12-14	830	sense+antisense	nuclear GFP-LacZ absent in >95% of cells
	myo-3::MtLS::gfp			**makes mitochondrial GFP in body muscle**	
	gfpG	exons 2-5	730	sense	mitochondrial GFP pattern of parent strain
				antisense	mitochondrial GFP pattern of parent strain
				sense+antisense	mitochondrial GFP absent in 98% of cells
	lacZL	exon 12-14	830	sense+antisense	mitochondrial GFP pattern of parent strain

Legend of Table 1

Each RNA was injected into 6-10 adult hermaphrodites (0.5-1x10^6 molecules into each gonad arm). After 4-6 hours (to clear pre-fertilized eggs from the uterus) injected animals were transferred and eggs collected for 20-22 hours. Progeny phenotypes were scored upon hatching and subsequently at 12-24 hour intervals.

a: To obtain a semi-quantitative assessment of the relationship between RNA dose and phenotypic response, we injected each *unc22A* RNA preparation at a series of different concentrations. At the highest dose tested (3.6x10^6 molecules per gonad), the

40 20-04-2005

PRINTED: 22-04-2005 TIPA 99910592

individual sense and antisense *unc22A* preparations produced some visible twitching (1% and 11% of progeny respectively). Comparable doses of ds-*unc22A* RNA produced visible twitching in all progeny, while a 120-fold lower dose of ds-*unc22A* RNA produced visible twitching in 30% of progeny.

b: *unc22C* also carries the intervening intron (43 nt).

c: *fem1A* also carries a portion (131 nt) of intron 10.

d: Animals in the first affected broods (laid at 4-24 hours after injection) showed movement defects indistinguishable from those of null mutants in *unc-54*. A variable fraction of these animals (25-75%) failed to lay eggs (another phenotype of *unc-54* null mutants), while the remainder of the paralyzed animals were egg-laying positive. This may indicate partial inhibition of *unc-54* activity in vulval muscles. Animals from later broods frequently exhibit a distinct partial loss-of-function phenotype, with contractility in a subset of body wall muscles.

e: Phenotypes of *hlh-1* inhibitory RNA include arrested embryos and partially elongated L1 larvae (the *hlh-1* null phenotype) seen in virtually all progeny from injection of ds-*hlh1A* and about half of the affected animals from ds-*hlh1B* and ds-*hlh1C*) and a set of less severe defects (seen with the remainder of the animals from ds-*hlh1B* and ds-*hlh1C*). The less severe phenotypes are characteristic of partial loss of function for *hlh-1*.

f: The host for these injections, PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ. This allows simultaneous assay for inhibition of *gfp* (loss of all fluorescence) and *lacZ* (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. ds-*gfpG* caused a loss of GFP in all but 0-3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0-5 additional bodywall muscles and in the eight vulval muscles.

20-04-2005

Printed: 22-04-2005 UPAT 99910592

Table 2. Effect of injection point on genetic inhibition in injected animals and their progeny.

dsRNA	Site of injection	Injected animal phenotype	Progeny Phenotype
None	gonad or body cavity	no twitching	no twitching
None	gonad or body cavity	strong nuclear & mitochondrial GFP	strong nuclear & mitochondrial GFP
unc22B	Gonad	weak twitchers	strong twitchers
unc22B	Body Cavity Head	weak twitchers	strong twitchers
unc22B	Body Cavity Tail	weak twitchers	strong twitchers
gfpG	Gonad	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
gfpG	Body Cavity Tail	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
lacZL	Gonad	lower nuclear GFP	rare or absent nuclear GFP
lacZL	Body Cavity Tail	lower nuclear GFP	rare or absent nuclear GFP

42 20-04-2005

Printed: 22-04-2005 HIRA 99910592

Table 3. *C. elegans* can respond in a gene-specific manner to environmental dsRNA.

Bacterial Food	Movement	Germline Phenotype	GFP-Transgene Expression
BL21(DE3)	0% twitch	< 1% female	< 1% faint GFP
BL21(DE3) [*fem-1* dsRNA]	0% twitch	43% female	< 1% faint GFP
BL21(DE3) [*unc22* dsRNA]	85% twitch	< 1% female	< 1% faint GFP
BL21(DE3) [*gfp* dsRNA]	0% twitch	< 1% female	12% faint GFP

Table 4. Effects of bathing *C. elegans* in a solution containing dsRNA.

dsRNA	Biological Effect
unc-22	Twitching (similar to partial loss of *unc-22* function)
pos-1	Embryonic arrest (similar to loss of *pos-1* function)
sqt-3	Shortened body (Dpy) (similar to partial loss of *sqt-3* function)

46 20-04-2005

Printed: 22-04-2005 JIPA1 99910592

In Table 2, gonad injections were carried out into the GFP reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ. This allowed simultaneous assay of inhibition with *gfp* (fainter overall fluorescence), *lacZ* (loss of nuclear fluorescence), and *unc-22* (twitching). Body cavity injections were carried out into the tail region, to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior region of the body cavity. An equivalent set of injections was also performed into a single gonad arm. For all sites of injection, the entire progeny brood showed phenotypes identical to those described in Table 1. This included progeny produced from both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could in part be due to the persistence of products already present in the injected adult. After ds-*unc22B* injection, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21/21) of these animals. Similar effects were seen with ds-*unc22A*. Injections of ds-*gfpG* or ds-*lacZL* produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of ds-*gfpG* and ds-*lacZL* produced no twitching, while injections of ds-*unc22A* produced no change in GFP fluorescence pattern.

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the present invention.

44 20-04-2005

Printed: 22-04-2005 TIRA 9991 0592

WE CLAIM:

1. A method to inhibit expression of a target gene in a cell comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA comprises a double-stranded structure with an identical nucleotide sequence as compared to a portion of the target gene.

2. The method of claim 1 in which the target gene is a cellular gene.

3. The method of claim 1 in which the target gene is an endogenous gene.

4. The method of claim 1 in which the target gene is a transgene.

5. The method of claim 1 in which the target gene is a viral gene.

6. The method of claim 1 in which the cell is from an animal.

7. The method of claim 1 in which the cell is from a plant.

8. The method of claim 6 in which the cell is from an invertebrate animal.

9. The method of claim 8 in which the cell is from a nematode.

10. The method of claim 1 in which the identical nucleotide sequence is at least 50 bases in length.

11. The method of claim 1 in which the target gene expression is inhibited by at least 10%.

12. The method of claim 1 in which the cell is present in an organism and inhibition of target gene expression demonstrates a loss-of function phenotype.

20-04-2005

Printed: 22-04-2005 TIPAT 99910592

13. The method of claim 1 in which the RNA comprises one strand which is self-complementary .

14. The method of claim 1 in which the RNA comprises two separate complementary strands.

15. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation outside the cell.

16. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation inside the cell.

17. The method of claim 1 in which the cell is present in an organism, and the RNA is introduced within a body cavity of the organism and outside the cell.

18. The method of claim 1 in which the cell is present in an organism and the RNA is introduced by extracellular injection into the organism.

19. The method of claim 1 in which the cell is present in a first organism, and the RNA is introduced to the first organism by feeding a second, RNA-containing organism to the first organism.

20. The method of claim 19 in which the second organism is engineered to produce an RNA duplex.

21. The method of claim 1 in which an expression construct in the cell produces the RNA.

22. A method to inhibit expression of a target gene comprising:

(a) providing an organism containing a target cell, wherein the target cell contains the target gene and the target gene is expressed in the target cell;

46 20-04-2005

(b) contacting a ribonucleic acid (RNA) with the organism, wherein the RNA is comprised of a double-stranded structure with duplexed ribonucleic acid strands and one of the strands is able to duplex with a portion of the target gene; and

(c) introducing the RNA into the target cell, thereby inhibiting expression of the target gene.

23. The method of claim 22 in which the organism is an animal.

24. The method of claim 22 in which the organism is a plant.

25. The method of claim 22 in which the organism is an invertebrate animal.

26. The method of claim 22 in which the organism is a nematode.

27. The method of claim 26 in which a formulation comprised of the RNA is applied on or adjacent to a plant, and disease associated with nematode infection of the plant is thereby reduced.

28. The method of claim 22 in which the identical nucleotide sequence is at least 50 nucleotides in length.

29. The method of claim 22 in which the expression of the target gene is inhibited by at least 10%.

30. The method of claim 22 in which the RNA is introduced within a body cavity of the organism and outside the target cell.

31. The method of claim 22 in which the RNA is introduced by extracellular injection into the organism.

Printed: 22-04-2005 HRAT 99910592

32. The method of claim 22 in which the organism is contacted with the RNA by feeding the organism food containing the RNA.

33. The method of claim 32 in which a genetically-engineered host transcribing the RNA comprises the food.

34. The method of claim 22 in which at least one strand of the RNA is produced by transcription of an expression construct.

35. The method of claim 35 in which the organism is a nematode and the expression construct is contained in a plant, and disease associated with nematode infection of the plant is thereby reduced.

36. A cell containing an expression construct,

wherein the expression construct transcribes at least one ribonucleic acid (RNA) and the RNA forms a double-stranded structure with duplexed strands of ribonucleic acid,

whereby said cell contains the double-stranded RNA structure and is able to inhibit expression of a target gene when the RNA is contacted with an organism containing the target gene.

37. A transgenic animal containing said cell of claim 36.

38. A transgenic plant containing said cell of claim 36.

39. A kit comprising reagents for inhibiting expression of a target gene in a cell,

wherein said kit comprises a means for introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, and

wherein the RNA has a double-stranded structure with an identical nucleotide sequence as compared to a portion of the target gene.

48 20-04-2005

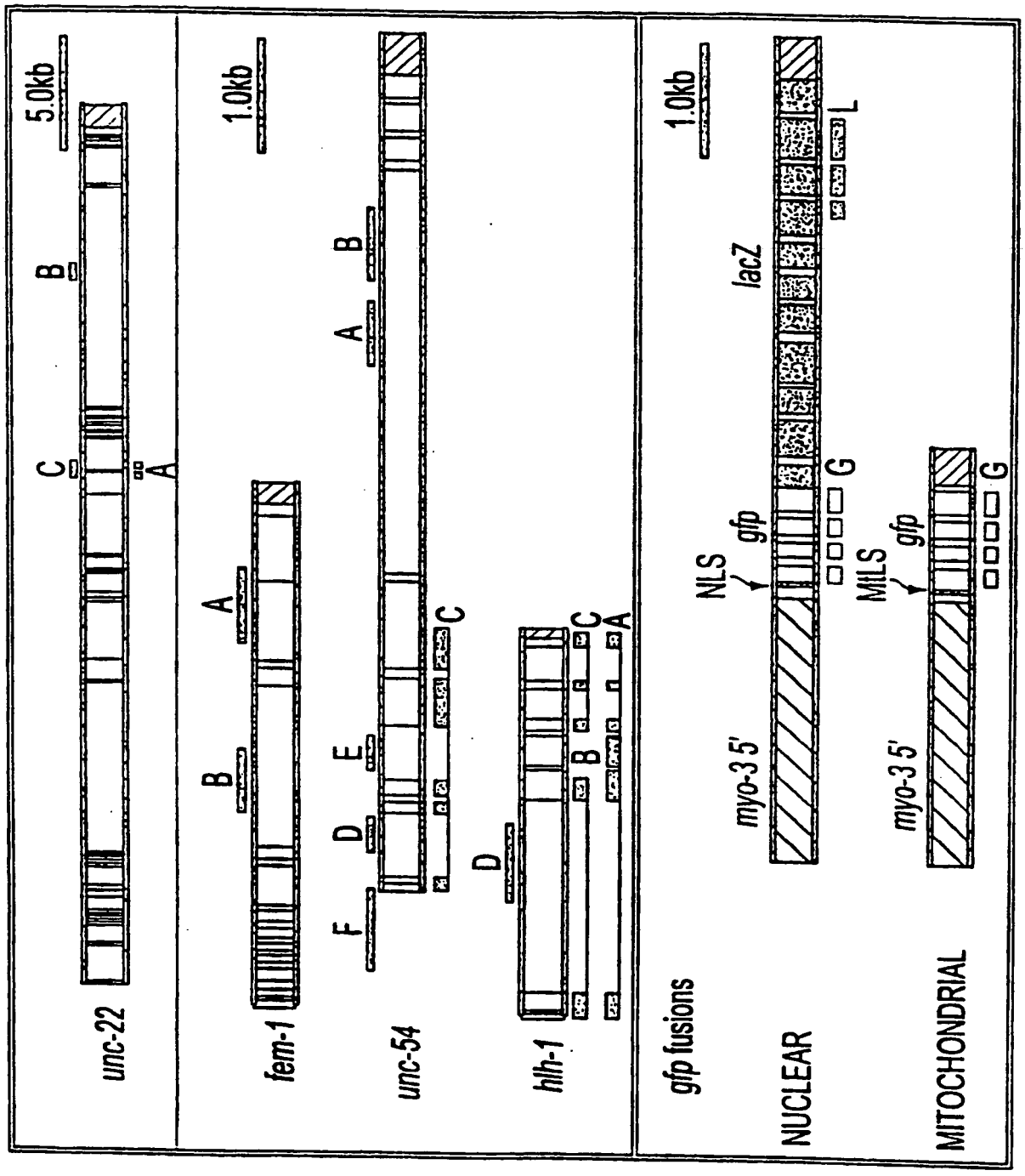

5.0kb
B
C
A
unc-22
1.0kb
A
B
fem-1
A
B
F
D
E
C
unc-54
D
B
C
A
hlh-1
1.0kb
gfp fusions
NLS
gfp
myo-3 5'
lacZ
L
G
NUCLEAR
MtLS
gfp
myo-3 5'
G
MITOCHONDRIAL

FIG. 1

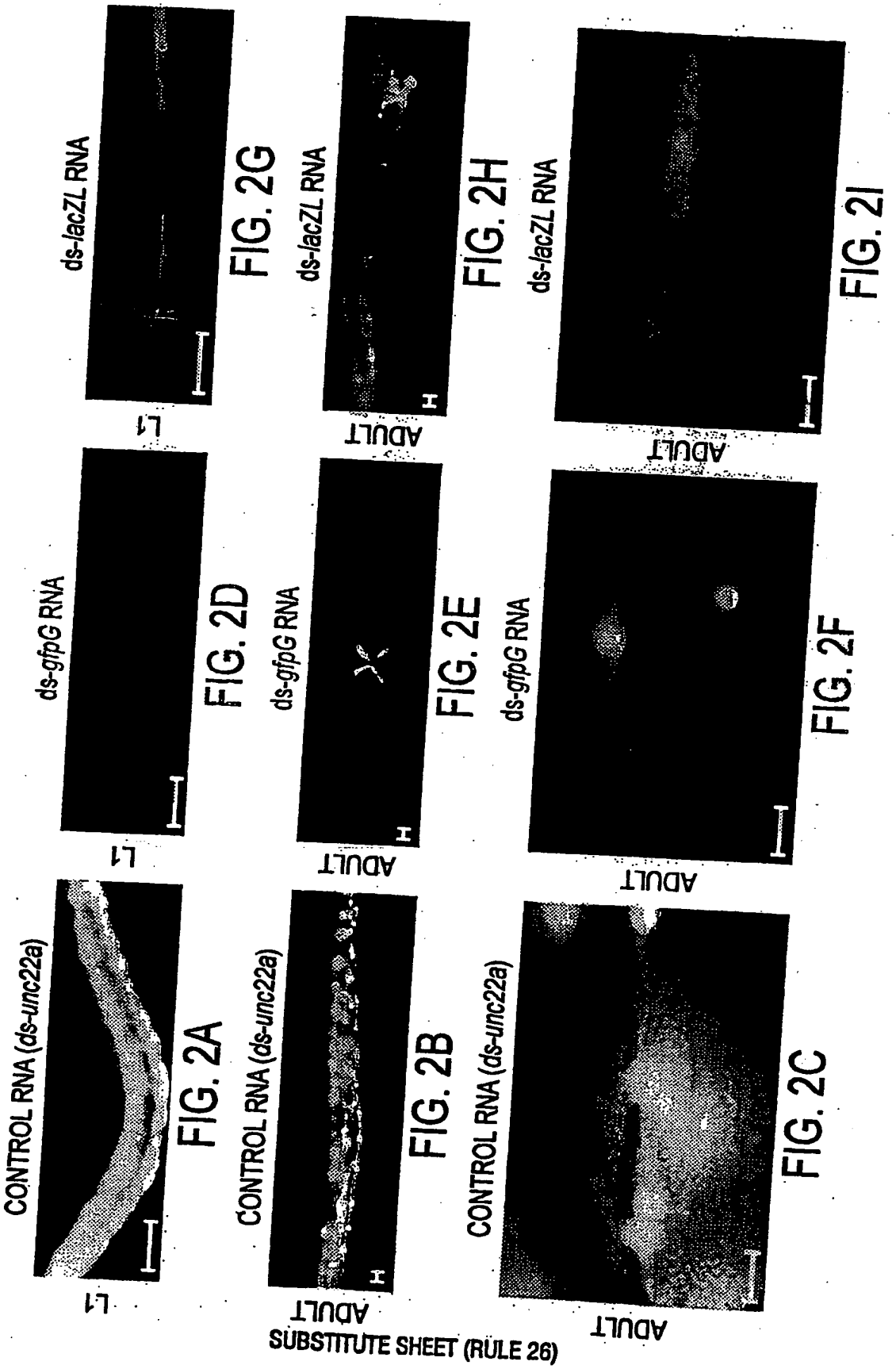


FIG. 2A FIG. 2B FIG. 2C
FIG. 2D FIG. 2E FIG. 2F
FIG. 2G FIG. 2H FIG. 2I

Printed: 22-04-2005

TRAT

99910592

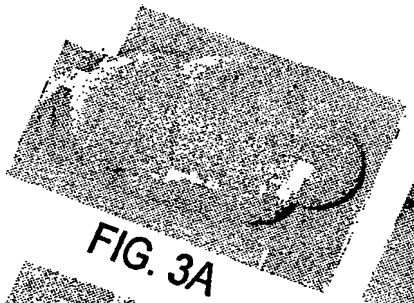


FIG. 3A

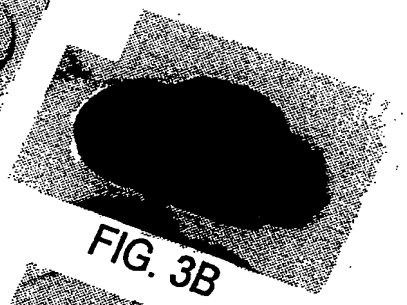


FIG. 3B

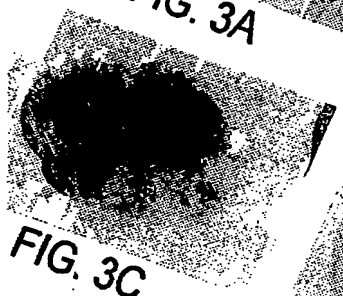


FIG. 3C

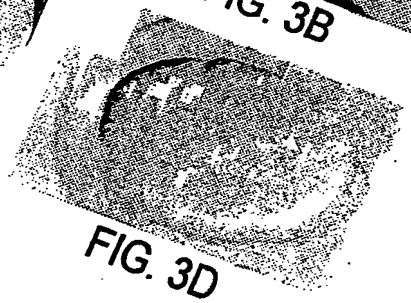


FIG. 3D

20-04-2005

Printed: 22-04-2005

TIPA

99910592

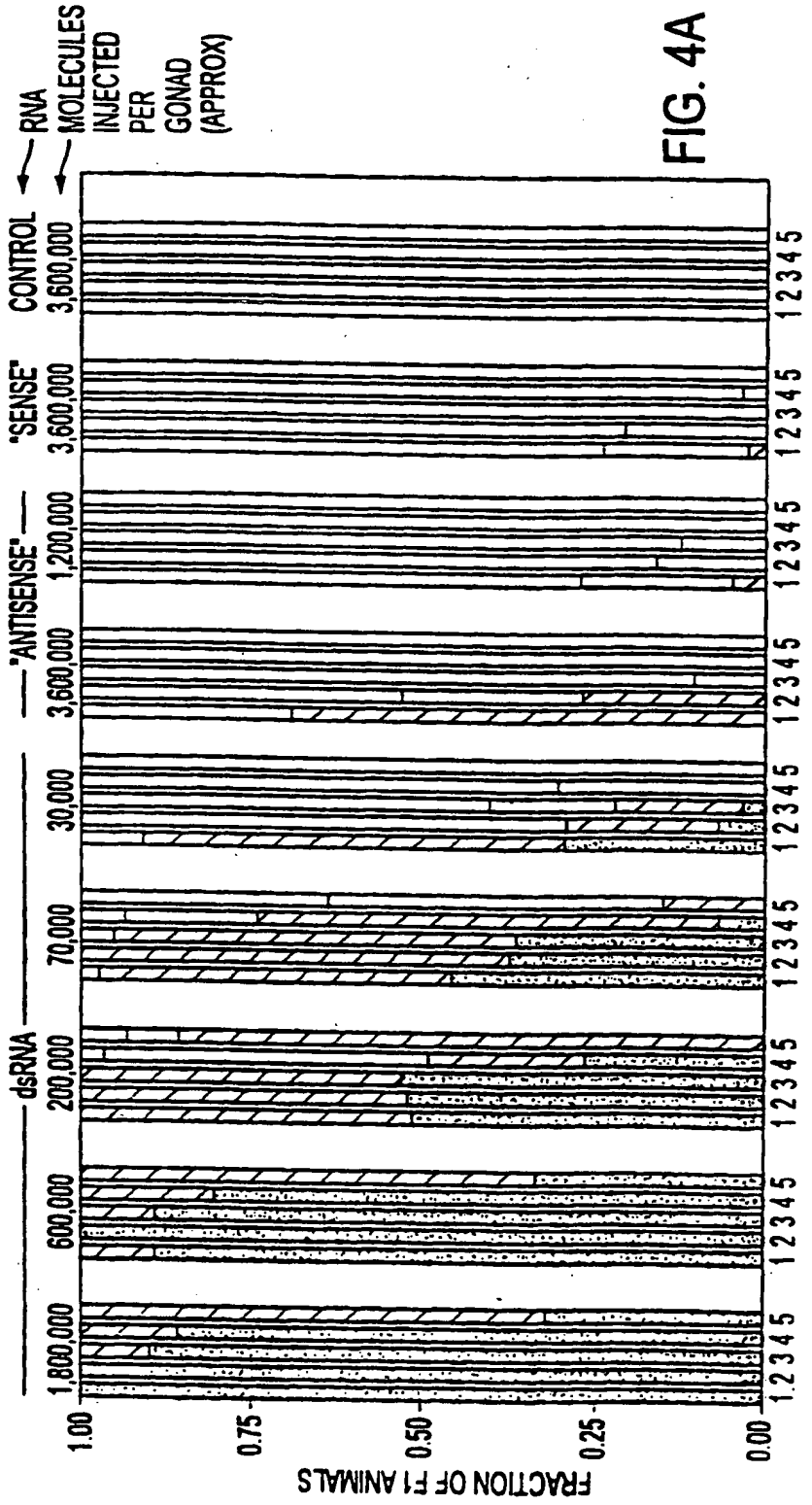


FIG. 4A

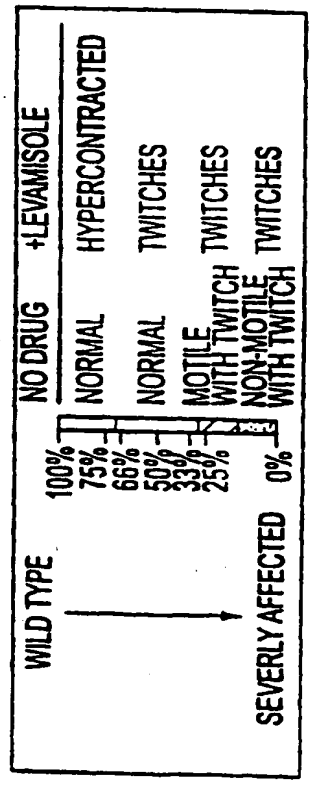


FIG. 4B

52

20-04-2005

Printed: 22-04-2005 EPA 99910592

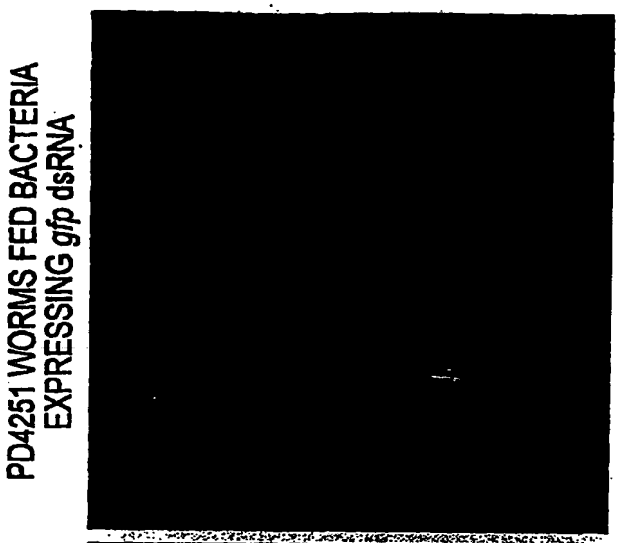


FIG. 5C

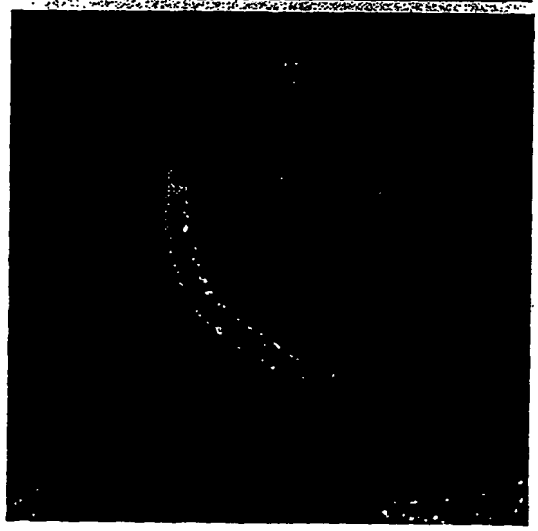


FIG. 5B

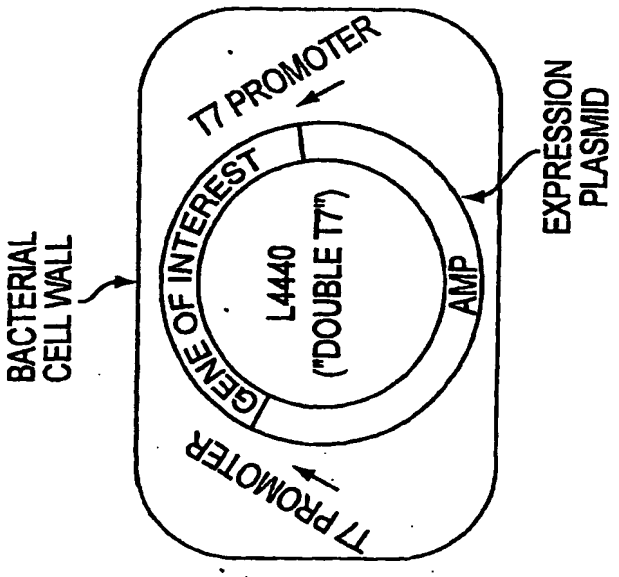


FIG. 5A

20-04-2005

Printed: 22-04-2005

TIPAT

99910592

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/27233

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C12N15/63 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RATCLIFF F ET AL: "A similarity between viral defense and gene silencing in plants" SCIENCE, vol. 276, no. 93, 6 June 1997, pages 1558-1560, XP002095874 see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C. [] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 May 1999	10/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ANDRES, S

Form PCT/ISA/210 (second sheet) (July 1992)

20-04-2005

Printed: 22-04-2005 EPA 99910592

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/27233

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FIRE, A. ET AL.: "Production of antisense RNA leads to effective and specific inhibition of gene expression in C. elegans muscle" DEVELOPMENT (CAMBRIDGE, UK) (1991), 113(2), 503-14, XP002103600 cited in the application see page 508, right-hand column, paragraph 2 see page 509, right-hand column - page 511, right-hand column see page 512, 'Discussion' and figure 7	1-39
A	MATZKE M A ET AL: "HOW AND WHY DO PLANTS INACTIVATE HOMOLOGOUS (TRANS)GENES?" PLANT PHYSIOLOGY, vol. 107, no. 3, 1 March 1995, pages 679-685, XP002021174 see page 680, left-hand column, paragraph 3 - right-hand column, paragraph 1 see page 682	1
P,X	FIRE A ET AL: "Potent and specific genetic interference by double - stranded RNA in Caenorhabditis elegans" NATURE, (1998 FEB 19) 391 (6669) 806-11., XP002095876 cited in the application see the whole document	1-3,6, 8-12, 14-18, 21-23, 25,26, 28-31, 34,39
P,X	MONTGOMERY M K ET AL: "Double - stranded RNA as a mediator in sequence-specific genetic silencing and co - suppression" TRENDS IN GENETICS, (1998 JUL) 14 (7) 255-8., XP004124680 cited in the application see the whole document	1-4, 6-12, 14-18, 36-39
P,X	TIMMONS L ET AL: "Specific interference by ingested dsRNA" NATURE, (1998 OCT 29) 395 (6705) 854., XP002103601 cited in the application see the whole document	1-3,6, 8-12, 14-23, 25,26, 28-34, 36,39

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

INTERNATIONAL SEARCH REPORT

...ernational application No.
PCT/US 98/ 27233

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 35 and claims 1-6,8-23,25-26,34 (as far as in vivo methods practised on animals are concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[] The additional search fees were accompanied by the applicant's protest.

[] No protest accompanied the payment of additional search fees.

56 20-04-2005

The following examples are meant to be illustrative of the present invention; however, the practice of the invention is not limited or restricted in any way by them.

METHODS

RNA Synthesis and Microinjection

RNA was synthesized from phagemid clones with T3 and T7 RNA polymerase[6], followed by template removal with two sequential DNase treatments. In cases where sense, antisense, and mixed RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original "sense" and "antisense" preparations. Nonetheless, RNA species accounting for less than 10% of purified RNA preparations would not have been observed. Without gel purification, the "sense" and "antisense" preparations produced significant interference. This interference activity was reduced or eliminated upon gel purification. By contrast, sense+antisense mixtures of gel purified and non-gel-purified RNA preparations produced identical effects.

Sense/antisense annealing was carried out in injection buffer[27] at 37°C for 10-30 minutes. Formation of predominantly double stranded material was confirmed by testing migration on a standard (non-denaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for double-stranded RNA of the appropriate length. Co-incubation of the two strands in a low-salt buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of double-stranded RNA *in vitro*. Non-annealed sense+antisense RNAs for *unc22B* and *gfpG* were tested for inhibitory RNA and found to be much more active than the individual single strands, but 2-4 fold less active than equivalent pre-annealed preparations.

After pre-annealing of the single strands for *unc22A*, the single electrophoretic species corresponding in size to that expected for dsRNA was purified using two rounds of gel electrophoresis. This material retained a high degree of inhibitory activity.

Except where noted, injection mixes were constructed so animals would receive an average of 0.5×10^6 to 1.0×10^6 molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections were compared with equal masses of RNA

(i.e., dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible; however, such variability would not affect any of the conclusions drawn herein.

Analysis of Phenotypes

Inhibition of endogenous genes was generally assayed in a wild type genetic background (N2). Features analyzed included movement, feeding, hatching, body shape, sexual identity, and fertility. Inhibition with *gfp* [27] and *lacZ* activity was assessed using strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccIs4251) made up of three plasmids: pSAK4 (*myo-3* promoter driving mitochondrially targeted GFP), pSAK2 (*myo-3* promoter driving a nuclear targeted GFP-LacZ fusion), and a *dpy-20* subclone[26] as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing a facile distinction between cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was performed by inserting the microinjection needle into the gonadal syncitium of adults and expelling 20-100 pl of solution (see reference 25). Body cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells was another effective means of RNA delivery, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16 hour intervals. This yields a series of semi-synchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short "clearance" interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. After the clearance period, individuals are produced which show the inhibitory phenotype.

Printed: 22-04-2005

TEXT

99910592

After injected animals have produced eggs for several days, gonads can in some cases "revert" to produce incompletely affected or phenotypically normal progeny.

The *unc-22* gene was chosen for initial comparisons of activity as a result of previous genetic analysis that yields a semi-quantitative comparison between *unc-22* gene activity and the movement phenotypes of animals[3,8]: decreases in activity produce an increasingly severe twitching phenotype, while complete loss of function results in the additional appearance of muscle structural defects and impaired motility. *unc-22* encodes an abundant but non-essential myofilament protein[7-9]. *unc-22* mRNA is present at several thousand copies per striated muscle cell[3].

Purified antisense and sense RNAs covering a 742 nt segment of *unc-22* had only marginal interference activity, requiring a very high dose of injected RNA for any observable effect (Table 1). By contrast, a sense-antisense mixture produced a highly effective inhibition of endogenous gene activity (Figure 4). The mixture was at least two orders of magnitude more effective than either single strand in inhibiting gene expression. The lowest dose of the sense-antisense mixture tested, approximately 60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. *unc-22* expression begins in embryos with approximately 500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent inhibitory activity of the sense-antisense mixture could reflect formation of double-stranded RNA (dsRNA), or conceivably some alternate synergy between the strands. Electrophoretic analysis indicated that the injected material was predominantly double stranded. The dsRNA was gel purified from the annealed mixture and found to retain potent inhibitory activity. Although annealing prior to injection was compatible with inhibition, it was not necessary. Mixing of sense and antisense RNAs in low salt (under conditions of minimal dsRNA formation), or rapid sequential injection of sense and antisense strands, were sufficient to allow complete inhibition. A long interval (>1 hour) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in inhibitory activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the complementary strand.

20-04-2005

Printed: 22-04-2005 IPAU 99910592

An issue of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic response mechanism[10]. Conceivably, our sense-antisense synergy could reflect a non-specific potentiation of antisense effects by such a panic mechanism. We found this not to be the case: co-injection of dsRNA segments unrelated to *unc-22* did not potentiate the ability of *unc-22* single strands to mediate inhibition. We also investigated whether double-stranded structure could potentiate interference activity when placed in *cis* to a single-stranded segment. No such potentiation was seen; unrelated double-stranded sequences located 5' or 3' of a single-stranded *unc-22* segment did not stimulate interference. Thus we have only observed potentiation of interference when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by *unc-22* dsRNA was specific. Progeny of injected animals exhibited behavior indistinguishable from characteristic *unc-22* loss of function mutants. We assessed target-specificity of dsRNA effects using three additional genes with well characterized phenotypes (Figure 1 and Table 1). *unc-54* encodes a body wall muscle myosin heavy chain isoform required for full muscle contraction [7,11,12], *fem-1* encodes an ankyrin-repeat containing protein required in hermaphrodites for sperm production[13,14], and *hlh-1* encodes a *C. elegans* homolog of the myoD family required for proper body shape and motility[15,16]. For each of these genes, injection of dsRNA produced progeny broods exhibiting the known null mutant phenotype, while the purified single strands produced no significant reduction in gene expression. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from inhibition of the corresponding gene. The exception (segment *unc54C*, which led to an embryonic and larval arrest phenotype not seen with *unc-54* null mutants) was illustrative. This segment covers the highly conserved myosin motor domain, and might have been expected to interfere with activity of other highly related myosin heavy chain genes[17]. This interpretation would support uses of the present invention in which nucleotide sequence comparison of dsRNA and target gene show less than 100% identity. The *unc54C* segment has been unique in our overall experience to date: effects of 18 other

20-04-2005

Printed: 22-04-2005 TPA 9940592

dsRNA segments have all been limited to those expected from characterized null mutants.

The strong phenotypes seen following dsRNA injection are indicative of inhibitory effects occurring in a high fraction of cells. The *unc-54* and *hlh-1* muscle phenotypes, in particular, are known to result from a large number of defective muscle cells[11,16]. To examine inhibition effects of dsRNA on a cellular level, we used a transgenic line expressing two different GFP-derived fluorescent reporter proteins in body muscle. Injection of dsRNA directed to *gfp* produced dramatic decreases in the fraction of fluorescent cells (Figure 2). Both reporter proteins were absent from the negative cells, while the few positive cells generally expressed both GFP forms.

The pattern of mosaicism observed with *gfp* inhibition was not random. At low doses of dsRNA, we saw frequent inhibition in the embryonically-derived muscle cells present when the animal hatched. The inhibitory effect in these differentiated cells persisted through larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically-derived striated muscles are born during early larval stages and were more resistant to inhibition. These cells have come through additional divisions (13-14 versus 8-9 for embryonic muscles[18,19]). At high concentrations of *gfp* dsRNA, we saw inhibition in virtually all striated bodywall muscles, with occasional single escaping cells including cells born in embryonic or postembryonic stages. The nonstriated vulval muscles, born during late larval development, appeared resistant to genetic inhibition at all tested concentrations of injected RNA. The latter result is important for evaluating the use of the present invention in other systems. First, it indicates that failure in one set of cells from an organism does not necessarily indicate complete non-applicability of the invention to that organism. Second, it is important to realize that not all tissues in the organism need to be affected for the invention to be used in an organism. This may serve as an advantage in some situations.

A few observations serve to clarify the nature of possible targets and mechanisms for RNA-mediated genetic inhibition in *C. elegans*:

First, dsRNA segments corresponding to a variety of intron and promoter sequences did not produce detectable inhibition (Table 1). Although consistent with

20-04-2005

Printed: 22-04-2005 UPAT 9991 0592

possible inhibition at a post-transcriptional level, these experiments do not rule out inhibition at the level of the gene.

Second, we found that dsRNA injection produces a dramatic decrease in the level of the endogenous mRNA transcript (Figure 3). Here, we targeted a *mex-3* transcript that is abundant in the gonad and early embryos[20], where straightforward *in situ* hybridization can be performed[5]. No endogenous *mex-3* mRNA was observed in animals injected with a dsRNA segment derived from *mex-3*. In contrast, injection of the purified *mex-3* antisense RNA resulted in animals that retained substantial endogenous mRNA levels (Figure 3D).

Third, dsRNA-mediated inhibition showed a surprising ability to cross cellular boundaries. Injection of dsRNA for *unc-22*, *gfp*, or *lacZ* into the body cavity of the head or tail produced a specific and robust inhibition of gene expression in the progeny brood (Table 2). Inhibition was seen in the progeny of both gonad arms, ruling out a transient "nicking" of the gonad in these injections. dsRNA injected into body cavity or gonad of young adults also produced gene-specific inhibition in somatic tissues of the injected animal (Table 2).

The effects described herein significantly augment available tools for studying gene function in *C. elegans* and other organisms. In particular, functional analysis should now be possible for a large number of interesting coding regions[21] for which no specific function have been defined. Several of these observations show the properties of dsRNA that may affect the design of processes for inhibition of gene expression. For example, we observed one case in which a nucleotide sequence shared between several myosin genes may inhibit gene expression of several members of a related gene family. This would not be a consideration for a target gene present in a single copy in the genome.

All books, articles and patents cited in this specification are incorporated herein by reference in their entirety.

REFERENCES

1. Izant, J. & Weintraub, H. *Cell* 36, 1007-1015 (1984).

2. Nellen, W. & Lichtenstein, C. *TIBS* 18, 419-423 (1993).

20-04-2005

Printed: 22-04-2005 TIPA 99910592

3. Fire, A., et al. *Development* 113, 503-514 (1991).

4. Guo, S. & Kemphues, K. *Cell* 81, 611-620 (1995).

5. Seydoux, G. & Fire, A. *Development* 120, 2823-2834 (1994).

6. Ausubel, F., et al. *Current Protocols in Molecular Biology*, John Wiley N.Y. (1990).

7. Brenner, S. *Genetics* 77, 71-94 (1974).

8. Moerman, D. & Baillie, D. *Genetics* 91, 95-104 (1979).

9. Benian, G., et al. *Genetics* 134, 1097-1104 (1993).

10. Proud, C. *TIBS* 20, 241-246 (1995).

11. Epstein H., et al. *J. Mol. Biol.* 90 291-300 (1974).

12. Karn, J., et al. *Proc. Natl. Acad. Sci. (U.S.A.)* 80, 4253-4257 (1983).

13. Doniach, T. & Hodgkin J.A. *Dev. Biol.* 106, 223-235 (1984).

14. Spence, A., et al. *Cell* 60, 981-990 (1990).

15. Krause, M., et al. *Cell* 63, 907-919 (1990).

16. Chen, L., et al. *Development*, 120, 1631-1641 (1994).

17. Dibb, N. J., et al. *J. Mol. Biol.* 205, 603-613 (1989).

18. Sulston, J., et al. *Dev. Biol.* 100, 64-119 (1983).

19. Sulston, J. & Horvitz, H. *Dev. Biol.* 82, 41-55 (1977).

20. Draper B.W., et al. *Cell* 87, 205-216 (1996).

21. Sulston, J., et al. *Nature* 356, 37-41 (1992).

22. Matzke, M. & Matzke, A. *Plant Physiol.* 107, 679-685 (1995).

23. Ratcliff, F., et al. *Science* 276, 1558-1560 (1997).

24. Latham, K. *Trends in Genetics* 12,134-138 (1996).

25. Mello, C. & Fire, A. *Methods in Cell Biology* 48, 451-482 (1995).

26. Clark, D., et al. *Mol. Gen. Genet.* 247, 367-378 (1995).

27. Chalfie, M., et al. *Science* 263, 802-805 (1994).

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

Table 1. Effects of sense, antisense, and mixed RNAs on progeny of injected animals.

Gene and Segment		Size	Injected RNA	F1 Phenotype
unc-22			***unc-22* null mutants: strong twitchers**[7,8]	
unc22A[a]	exon 21-22	742	sense	wild type
			antisense	wild type
			sense+antisense	strong twitchers (100%)
unc22B	exon 27	1033	sense	wild type
			antisense	wild type
			sense+antisense	strong twitchers (100%)
unc22C	exon 21-22[b]	785	sense+antisense	strong twitchers (100%)
fem-1			***fem-1* null mutants: female (no sperm)**[13]	
fem1A	exon 10[c]	531	sense	hermaphrodite (98%)
			antisense	hermaphrodite (>98%)
			sense+antisense	female (72%)
fem1B	intron 8	556	sense+antisense	hermaphrodite (>98%)
unc-54			***unc-54* null mutants: paralyzed**[7,11]	
unc54A	exon 6	576	sense	wild type (100%)
			antisense	wild type (100%)
			sense+antisense	paralyzed (100%)[d]
unc54B	exon 6	651	sense	wild type (100%)
			antisense	wild type (100%)
			sense+antisense	paralyzed (100%)[d]
unc54C	exon 1-5	1015	sense+antisense	arrested embryos and larvae (100%)
unc54D	promoter	567	sense+antisense	wild type (100%)
unc54E	intron 1	369	sense+antisense	wild type (100%)
unc54F	intron 3	386	sense+antisense	wild type (100%)

86 20-04-2005

Printed: 22-04-2005
TIPAT
99910593

Table 1 (continued).

	Gene and Segment		Size	Injected RNA	F1 Phenotype
	hlh-1			***hlh-1* null mutants: lumpy-dumpy larvae**[16]	
	hlh1A	exons 1-6	1033	sense	wild type (<2% lpy-dpy)
				antisense	wild type (<2% lpy-dpy)
				sense+antisense	lpy-dpy larvae (>90%)[e]
	hlh1B	exons 1-2	438	sense+antisense	lpy-dpy larvae (>80%)[e]
	hlh1C	exons 4-6	299	sense+antisense	lpy-dpy larvae (>80%)[e]
	hlh1D	intron 1	697	sense+antisense	wild type (<2% lpy-dpy)
	myo-3 driven GFP transgenes [f]				
	myo-3::NLS::gfp::lacZ			**makes nuclear GFP in body muscle**	
	gfpG	exons 2-5	730	sense	nuclear GFP-LacZ pattern of parent strain
				antisense	nuclear GFP-LacZ pattern of parent strain
				sense+antisense	nuclear GFP-LacZ absent in 98% of cells
	lacZL	exon 12-14	830	sense+antisense	nuclear GFP-LacZ absent in >95% of cells
	myo-3::MtLS::gfp			**makes mitochondrial GFP in body muscle**	
	gfpG	exons 2-5	730	sense	mitochondrial GFP pattern of parent strain
				antisense	mitochondrial GFP pattern of parent strain
				sense+antisense	mitochondrial GFP absent in 98% of cells
	lacZL	exon 12-14	830	sense+antisense	mitochondrial GFP pattern of parent strain

Legend of Table 1

Each RNA was injected into 6-10 adult hermaphrodites (0.5-1x10^6 molecules into each gonad arm). After 4-6 hours (to clear pre-fertilized eggs from the uterus) injected animals were transferred and eggs collected for 20-22 hours. Progeny phenotypes were scored upon hatching and subsequently at 12-24 hr intervals.

29-04-2005

a: To obtain a semi-quantitative assesment of the relationship between RNA dose and phenotypic response, we injected each *unc22A* RNA preparation at a series of different concentrations. At the highest dose tested ($3.6x10^6$ molecules per gonad), the individual sense and antisense *unc22A* preparations produced some visible twitching (1% and 11% of progeny respectively). Comparable doses of ds-*unc22A* RNA produced visible twitching in all progeny, while a 120-fold lower dose of ds-*unc22A* RNA produced visible twitching in 30% of progeny.

b: *unc22C* also carries the intervening intron (43 nt).

c: *fem1A* also carries a portion (131 nt) of intron 10.

d: Animals in the first affected broods (layed at 4-24 hours after injection) showed movement defects indistinguishable from those of null mutants in *unc-54*. A variable fraction of these animals (25-75%) failed to lay eggs (another phenotype of *unc-54* null mutants), while the remainder of the paralyzed animals were egg-laying positive. This may indicate partial inhibition of *unc-54* activity in vulval muscles. Animals from later broods frequently exhibit a distinct partial loss-of-function phenotype, with contractility in a subset of body wall muscles.

e: Phenotypes of *hlh-1* inhibitory RNA include arrested embryos and partially elongated L1 larvae (the *hlh-1* null phenotype) seen in virtually all progeny from injection of ds-*hlh1A* and about half of the affected animals from ds-*hlh1B* and ds-*hlh1C*) and a set of less severe defects (seen with the remainder of the animals from ds-*hlh1B* and ds-*hlh1C*). The less severe phenotypes are characteristic of partial loss of function for *hlh-1*.

f: The host for these injections, PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ. This allows simultaneous assay for inhibition of *gfp* (loss of all fluorescence) and *lacZ* (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. ds-*gfpG* caused a loss of GFP in all but 0-3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0-5 additional bodywall muscles and in the eight vulval muscles.

Table 2. Effect of injection point on genetic inhibition in injected animals and their progeny.

dsRNA	Site of injection	Injected animal phenotype	Progeny Phenotype
None	gonad or body cavity	no twitching	no twitching
None	gonad or body cavity	strong nuclear & mitochondrial GFP	strong nuclear & mitochondrial GFP
unc22B	Gonad	weak twitchers	strong twitchers
unc22B	Body Cavity Head	weak twitchers	strong twitchers
unc22B	Body Cavity Tail	weak twitchers	strong twitchers
gfpG	Gonad	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
gfpG	Body Cavity Tail	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
lacZL	Gonad	lower nuclear GFP	rare or absent nuclear GFP
lacZL	Body Cavity Tail	lower nuclear GFP	rare or absent nuclear GFP

Gonad injections were carried out into the GFP reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ. This allowed simultaneous assay of inhibition with *gfp* (fainter overall fluorescence), *lacZ* (loss of nuclear fluorescence), and *unc-22* (twitching). Body cavity injections were carried out into the tail region, to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior region of the body cavity. An equivalent set of injections was also performed into a single gonad arm. For all sites of injection, the entire progeny brood showed phenotypes identical to those described in Table 1. This included progeny produced from both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could in part be due to the persistance of products already present in the injected adult. After ds-*unc22B* injection, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21/21) of these animals. Similar effects were seen with ds-*unc22A*. Injections of ds-*gfpG* or ds-*lacZL* produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of ds-*gfpG* and ds-*lacZL* produced no twitching, while injections of ds-*unc22A* produced no change in GFP fluorescence pattern.

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the present invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the present invention.

Printed:22-04-2005 IPAU 99911059 2

WE CLAIM:

1. A method to inhibit expression of a target gene in a cell comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA has a double-stranded structure with an identical nucleotide sequence as compared to a portion of the target gene.

2. The method of claim 1 in which the target gene is a cellular gene.

3. The method of claim 1 in which the target gene is an endogenous gene.

4. The method of claim 1 in which the target gene is a transgene.

5. The method of claim 1 in which the target gene is a viral gene.

6. The method of claim 1 in which the cell is from an animal.

7. The method of claim 1 in which the cell is from a plant.

8. The method of claim 6 in which the cell is from an invertebrate animal.

9. The method of claim 8 in which the cell is from a nematode.

10. The method of claim 1 in which the identical nucleotide sequence is at least 50 nucleotides in length.

11. The method of claim 1 in which the target gene expression is inhibited by at least 10%.

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

12. The method of claim 1 in which the cell is present in an organism and inhibition of target gene expression demonstrates a loss-of function phenotype.

13. The method of any one of claims 1-12 in which the RNA has one self-complementary strand.

14. The method of any one of claims 1-12 in which the RNA has two separate complementary strands.

15. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation outside the cell.

16. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation inside the cell.

17. The method of any one of claims 1-12 in which the RNA has no single-stranded structure.

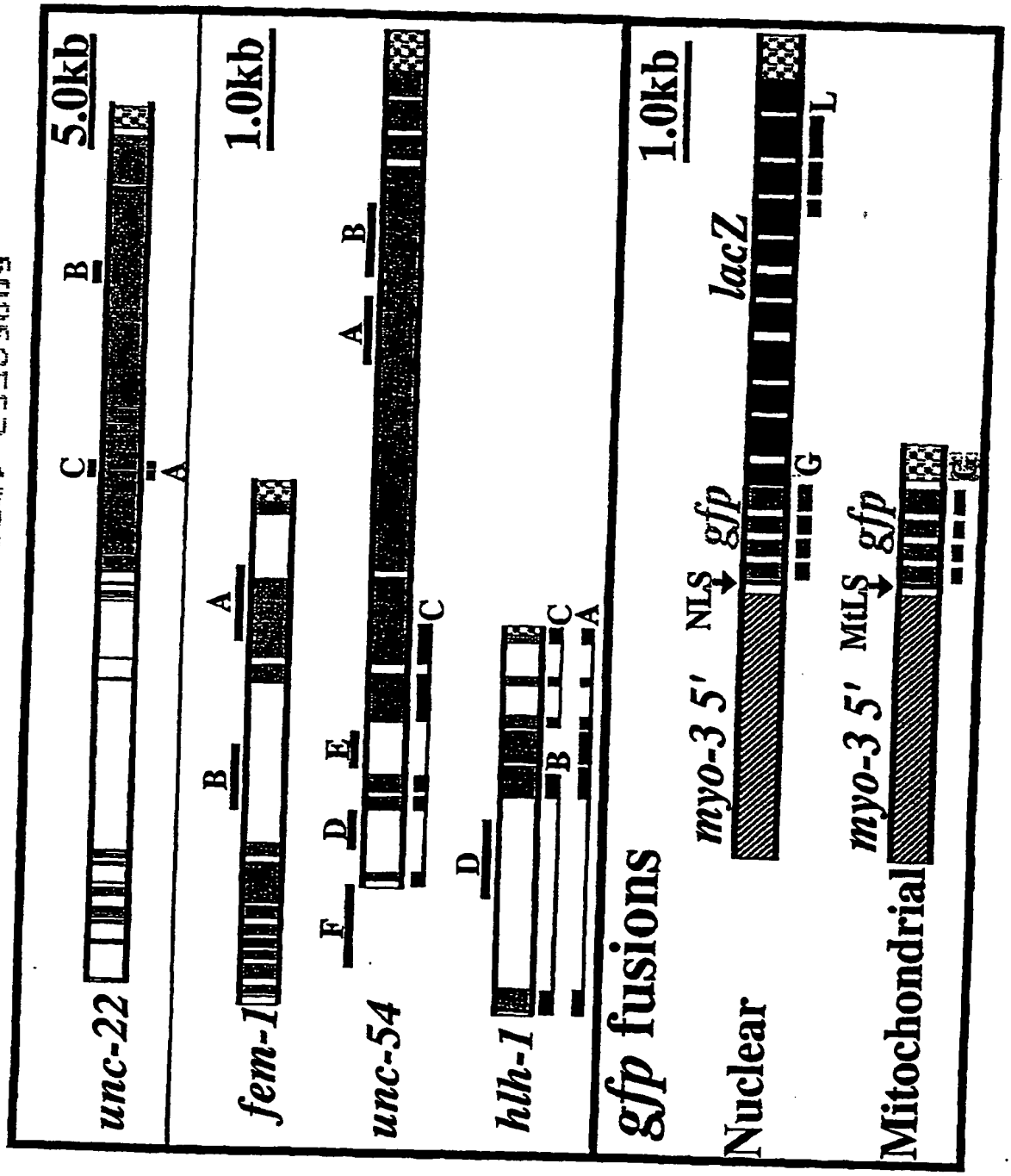
18. The method of any one of claims 1-12 in which the RNA is introduced within a body cavity of an animal and outside the cell.

19. The method of any one of claims 1-12 in which the RNA is introduced by extracellular injection into a body cavity of an organism.

20. The method of any one of claims 1-12 in which an expression vector in a cell produces the RNA.

69 20-04-2005

FIGURE 1


unc-22
5.0kb
C
B
A
fem-1
1.0kb
B
A
unc-54
F
D
E
A
B
C
hlh-1
D
B
C
A
gfp fusions
1.0kb
Nuclear
myo-3 5'
NLS
gfp
lacZ
G
L
Mitochondrial
myo-3 5'
MtLS
gfp
G

Eingang 22.04.2005

TIPA

99910592

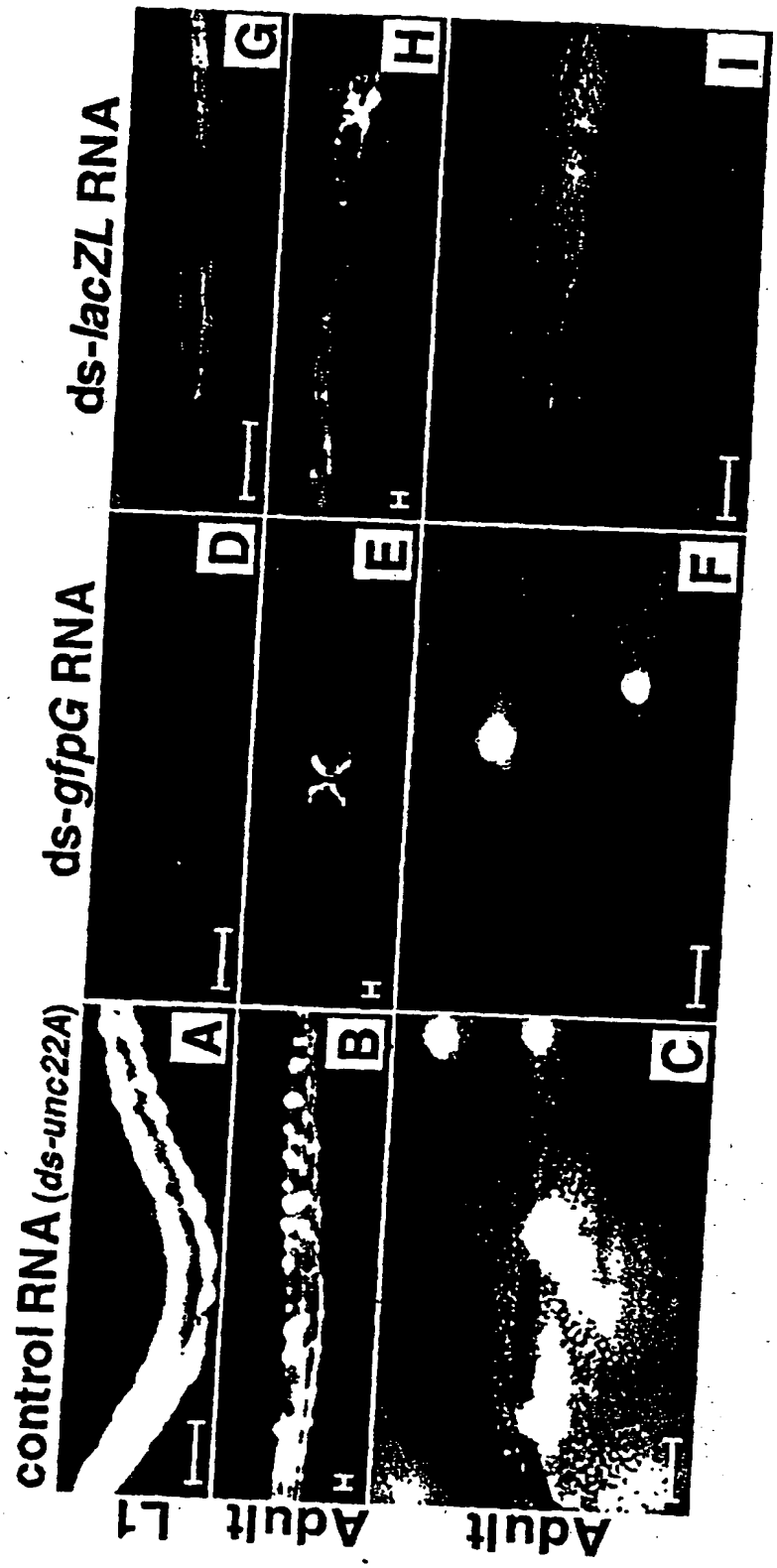


FIGURE 2

92

20.04.2005

20-04-2005

TEXT

99910592

FIGURE 3

20-04-2005



20-04-2005

26C227 29589003

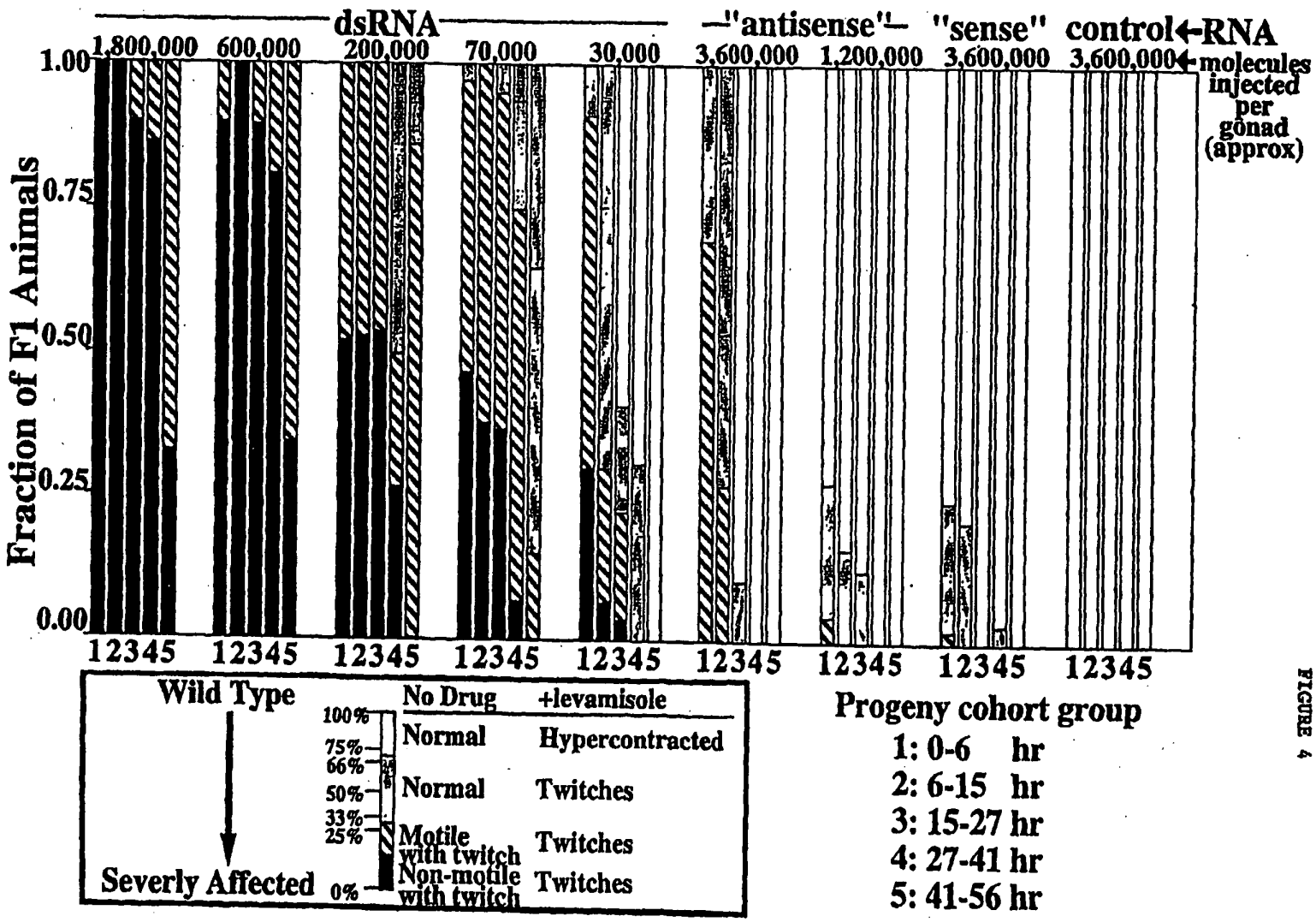


FIGURE 4

Printed: 22-04-2005

EPO - DG 1

08. 2000

(43)

PCT/US98/27233
98964202.0

REC'D 02 MAR 1999
WIPO PCT

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

February 26, 1999

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *09/215,257*
FILING DATE: *December 18, 1998*
PCT APPLICATION NUMBER: *PCT/US98/27233*

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

P. SWAIN
Certifying Officer

22-04-2005

Printed: 20-04-2005 TIPA1 99910592

APPLICATION UNDER UNITED STATES PATENT LAWS

Invention: GENETIC INHIBITION BY DOUBLE-STRANDED RNA

Inventor (s): Andrew FIRE, Si Q. XU, Mary K. MONTGOMERY, Stephen A. KOSTAS, Lisa TIMMONS, Hiroaki TABARA, Samuel E. DRIVER, and Craig C. MELLO

Pillsbury Madison & Sutro LLP
Intellectual Property Group
1100 New York Avenue, N.W.
Ninth Floor, East Tower
Washington, D.C. 20005-3918
Attorneys
Telephone: (202) 861-3000

This is a:

- ☐ Provisional Application
- ☒ Regular Utility Application
- ☐ Continuing Application
- ☐ PCT National Phase Application
- ☐ Design Application
- ☐ Reissue Application
- ☐ Plant Application
- ☐ Substitute Specification
 Sub. Spec Filed _____
 in App. No. /

SPECIFICATION

09215257 .121098

GENETIC INHIBITION BY DOUBLE-STRANDED RNA

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Appln. No. 60/068,562, filed December 23, 1997.

GOVERNMENT RIGHTS

This invention was made with U.S. government support under grant numbers GM-37706, GM-17164, HD-33769 and GM-07231 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to gene-specific inhibition of gene expression by double-stranded ribonucleic acid (dsRNA).

2. Description of the Related Art

Targeted inhibition of gene expression has been a long-felt need in biotechnology and genetic engineering. Although a major investment of effort has been made to achieve this goal, a more comprehensive solution to this problem was still needed.

Classical genetic techniques have been used to isolate mutant organisms with reduced expression of selected genes. Although valuable, such techniques require laborious mutagenesis and screening programs, are limited to organisms in which genetic manipulation is well established (e.g., the existence of selectable markers, the ability to control genetic segregation and sexual reproduction), and are limited to applications in which a large number of cells or organisms can be sacrificed to isolate the desired mutation. Even under these circumstances, classical genetic techniques can fail to produce mutations in specific target genes of interest, particularly when complex genetic pathways are involved. Many applications of molecular genetics require the ability to go beyond classical genetic screening techniques and efficiently produce a *directed* change in gene expression in a specified group of cells or organisms. Some such applications are knowledge-based projects in which it is of importance to understand what effects the loss of a specific gene product (or products) will have on the behavior of the cell or organism. Other applications are engineering based, for example: cases in which is important to

Printed: 22-04-2005 IPAU 99910592

produce a population of cells or organisms in which a specific gene product (or products) has been reduced or removed. A further class of applications is therapeutically based in which it would be valuable for a functioning organism (e.g., a human) to reduce or remove the amount of a specified gene product (or products). Another class of applications provides a disease model in which a physiological function in a living organism is genetically manipulated to reduce or remove a specific gene product (or products) without making a permanent change in the organism's genome.

In the last few years, advances in nucleic acid chemistry and gene transfer have inspired new approaches to engineer specific interference with gene expression. These approaches are described below.

Use of Antisense Nucleic Acids to Engineer Interference

Antisense technology has been the most commonly described approach in protocols to achieve gene-specific interference. For antisense strategies, stochiometric amounts of single-stranded nucleic acid complementary to the messenger RNA for the gene of interest are introduced into the cell. Some difficulties with antisense-based approaches relate to delivery, stability, and dose requirements. In general, cells do not have an uptake mechanism for single-stranded nucleic acids, hence uptake of unmodified single-stranded material is extremely inefficient. While waiting for uptake into cells, the single-stranded material is subject to degradation. Because antisense interference requires that the interfering material accumulate at a relatively high concentration (at or above the concentration of endogenous mRNA), the amount required to be delivered is a major constraint on efficacy. As a consequence, much of the effort in developing antisense technology has been focused on the production of modified nucleic acids that are both stable to nuclease digestion and able to diffuse readily into cells. The use of antisense interference for gene therapy or other whole-organism applications has been limited by the large amounts of oligonucleotide that need to be synthesized from non-natural analogs, the cost of such synthesis, and the difficulty even with high doses of maintaining a sufficiently concentrated and uniform pool of interfering material in each cell.

09215257 - 121608

98 20-04-2005

20-04-2005 TIRAT 99910592

Triple-Helix Approaches to Engineer Interference

A second, proposed method for engineered interference is based on a triple helical nucleic acid structure. This approach relies on the rare ability of certain nucleic acid populations to adopt a triple-stranded structure. Under physiological conditions, nucleic acids are virtually all single- or double-stranded, and rarely if ever form triple-stranded structures. It has been known for some time, however, that certain simple purine- or pyrimidine-rich sequences could form a triple-stranded molecule *in vitro* under extreme conditions of pH (i.e., in a test tube). Such structures are generally very transient under physiological conditions, so that simple delivery of unmodified nucleic acids designed to produce triple-strand structures does not yield interference. As with antisense, development of triple-strand technology for use *in vivo* has focused on the development of modified nucleic acids that would be more stable and more readily absorbed by cells *in vivo*. An additional goal in developing this technology has been to produce modified nucleic acids for which the formation of triple-stranded material proceeds effectively at physiological pH.

09215257 .12198

Co-Suppression Phenomena and Their Use in Genetic Engineering

A third approach to gene-specific interference is a set of operational procedures grouped under the name "co-suppression". This approach was first described in plants and refers to the ability of transgenes to cause silencing of an unlinked but homologous gene. More recently, phenomena similar to co-suppression have been reported in two animals: *C. elegans* and *Drosophila*. Co-suppression was first observed by accident, with reports coming from groups using transgenes in attempts to achieve over-expression of a potentially useful locus. In some cases the over-expression was successful while, in many others, the result was opposite from that expected. In those cases, the transgenic plants actually showed less expression of the endogenous gene. Several mechanisms have so far been proposed for transgene-mediated co-suppression in plants; all of these mechanistic proposals remain hypothetical, and no definitive mechanistic description of the process has been presented. The models that have been proposed to explain co-suppression can be placed in two different categories. In one set of proposals, a direct physical interaction at the DNA- or chromatin-level between two different chromosomal sites has been

20-04-2005

PCT/EP 22-04-2005 TIPAT 99910592

hypothesized to occur; an as-yet-unidentified mechanism would then lead to *de novo* methylation and subsequent suppression of gene expression. Alternatively, some have postulated an RNA intermediate, synthesized at the transgene locus, which might then act to produce interference with the endogenous gene. The characteristics of the interfering RNA, as well as the nature of the interference process, have not been determined. Recently, a set of experiments with RNA viruses have provided some support for the possibility of RNA intermediates in the interference process. In these experiments, a replicating RNA virus is modified to include a segment from a gene of interest. This modified virus is then tested for its ability to interfere with expression of the endogenous gene. Initial results with this technique have been encouraging, however, the properties of the viral RNA that are responsible for interference effects have not been determined and, in any case, would be limited to plants which are hosts of the plant virus.

09215257.121898

Distinction between the Present Invention and Antisense Approaches

The present invention differs from antisense-mediated interference in both approach and effectiveness. Antisense-mediated genetic interference methods have a major challenge: delivery to the cell interior of specific single-stranded nucleic acid molecules at a concentration that is equal to or greater than the concentration of endogenous mRNA. Double-stranded RNA-mediated inhibition has advantages both in the stability of the material to be delivered and the concentration required for effective inhibition. Below, we disclose that in the model organism *C. elegans*, the present invention is at least 100-fold more effective than an equivalent antisense approach (i.e., dsRNA is at least 100-fold more effective than the injection of purified antisense RNA in reducing gene expression). These comparisons also demonstrate that inhibition by double-stranded RNA must occur by a mechanism distinct from antisense interference.

Distinction between the Present Invention and Triple-Helix Approaches

The limited data on triple strand formation argues against the involvement of a stable triple-strand intermediate in the present invention. Triple-strand structures occur rarely, if at all, under physiological conditions and are limited to very unusual base sequence with long runs of purines and pyrimidines. By contrast, dsRNA-mediated

20-04-2005

PAGE 01/22/04 2005 TIPA 99910592

inhibition occurs efficiently under physiological conditions, and occurs with a wide variety of inhibitory and target nucleotide sequences. The present invention has been used to inhibit expression of 18 different genes, providing phenocopies of null mutations in these genes of known function. The extreme environmental and sequence constraints on triple-helix formation make it unlikely that dsRNA-mediated inhibition *in C. elegans* is mediated by a triple-strand structure.

Distinction between Present Invention and Co-Suppression Approaches

The transgene-mediated genetic interference phenomenon called co-suppression may include a wide variety of different processes. From the viewpoint of application to other types of organisms, the co-suppression phenomenon in plants is difficult to extend. A confounding aspect in creating a general technique based on co-suppression is that some transgenes in plants lead to suppression of the endogenous locus and some do not. Results in *C. elegans* and *Drosophila* indicate that certain transgenes can cause interference (i.e., a quantitative decrease in the activity of the corresponding endogenous locus) but that most transgenes do not produce such an effect. The lack of a predictable effect in plants, nematodes, and insects greatly limits the usefulness of simply adding transgenes to the genome to interfere with gene expression. Viral-mediated co-suppression in plants appears to be quite effective, but has a number of drawbacks. First, it is not clear what aspects of the viral structure are critical for the observed interference. Extension to another system would require discovery of a virus in that system which would have these properties, and such a library of useful viral agents are not available for many organisms. Second, the use of a replicating virus within an organism to effect genetic changes (e.g., long- or short-term gene therapy) requires considerably more monitoring and oversight for deleterious effects than the use of a defined nucleic acid as in the present invention.

The present invention avoids the disadvantages of the previously-described methods for genetic interference. Several advantages of the present invention are discussed below, but numerous others will be apparent to one of ordinary skill in the biotechnology and genetic engineering arts.

09851271 .252504 60

20-04-2005

Printed: 22-04-2005 TIPAT 99910592

09215257.121698

SUMMARY OF THE INVENTION

A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

The RNA may comprise one or more strands of polymerized ribonucleotide; it may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. RNA containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be

102 20-04-2005

Received 22-04-2005
EPO
99910592

defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or in *vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region may be used to transcribe the RNA strand (or strands).

The RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

The advantages of the present invention include: the ease of introducing double-stranded RNA into cells, the low concentration of RNA which can be used, the stability of double-stranded RNA, and the effectiveness of the inhibition. The ability to use a low concentration of a naturally-occurring nucleic acid avoids several disadvantages of antisense interference. This invention is not limited to *in vitro* use or to specific sequence compositions, as are techniques based on triple-strand formation. And unlike antisense interference, triple-strand interference, and co-suppression, this invention does not suffer from being limited to a particular set of target genes, a particular portion of the target gene's nucleotide sequence, or a particular transgene or viral delivery method. These concerns have been a serious obstacle to designing general strategies according to the prior art for inhibiting gene expression of a target gene of interest.

Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease

0221557 EP 04 8748

Printed: 22-04-2005 EPA 99910592

models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; *unc-22* [9], *unc-54* [12], *fem-1* [14], and *hlh-1* [15]).

Figures 2 A-I show analysis of inhibitory RNA effects in individual cells. These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins, nuclear GFP-LacZ and mitochondrial GFP. The micrographs show progeny of injected animals visualized by a fluorescence microscope. Panels A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (ds-*unc22A*). Panels D-F show progeny of animals injected with ds-*gfpG*. Panels G-I demonstrate specificity. Animals are injected with ds-*lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. Panel H shows a typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars are 20 μm.

Figures 3 A-D show effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA. Micrographs show *in situ* hybridization to embryos (dark stain). Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous *mex-3* RNA[20]). Panel C: Embryo from a parent injected with purified *mex-3*B antisense RNA. These embryos and the parent animals retain the *mex-3* mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with dsRNA corresponding to *mex-3*B; no *mex-3* RNA was detected. Scale: each embryo is approximately 50 μm in length.

Figure 4 shows inhibitory activity of *unc-22A* as a function of structure and concentration. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included. Progeny cohort groups are labeled 1 for 0-6 hours, 2 for 6-15

002155574.1200

1024 20-04-2005

22-04-2005 IPEA 99910592

hours, 3 for 15-27 hours, 4 for 27-41 hours, and 5 for 41-56 hours The bottom-left diagram shows genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids[8,3].

Figures 5 A-C show examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. Panel A: General strategy for production of dsRNA by cloning a segment of interest between flanking copies of the bacteriophage T7 promoter and transcribing both strands of the segment by transfecting a bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter. Panel B: A GFP-expressing *C. elegans* strain, PD4251 (see Figure 2), fed on a native bacterial host. Panel C: PD4251 animals reared on a diet of bacteria expressing dsRNA corresponding to the coding region for *gfp*.

WO 00/01846 PCT/GB99/02152

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing sequence-specific inhibition of gene expression by introducing double-stranded RNA (dsRNA). A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell. Inhibition is sequence-specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell (i.e., a cellular gene), an endogenous gene (i.e., a cellular gene present in the genome), a transgene (i.e., a gene construct inserted at an ectopic site in the genome of the cell), or a gene from a pathogen which is capable of infecting an organism from which the cell is derived. Depending on the particular target gene and the dose of double stranded RNA material delivered, this process may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The

20-04-2005

Printed 22-04-2005 IPA 99910592

consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

0021525/1248 PCT/EP03/50598

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

The RNA may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored

106 20-04-2005

PATENT
99910592

to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

RNA containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, *Sequence Analysis Primer*, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases.

As disclosed herein, 100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of

09215257-121898

being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis; field crops (e.g., alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative generae of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tfhchonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphalenchus, Criconemella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, and Xiphinema). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

09215257 - 12 APR 2005

EPO - DG 1 22.04.2005 11FA1 99910592

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or in *vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art[32, 33, 34] (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by *in vitro* enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, intro-

09215257 .121805

19 20-04-2005

duced orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. For example, the RNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. We disclose herein that in *C. elegans*, double-stranded RNA introduced outside the cell inhibits gene expression. Vascular or extravascular circulation, the blood or lymph system, the phloem, the roots, and the cerebrospinal fluid are sites where the RNA may be introduced. A transgenic organism that expresses RNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.

Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

The present invention may be used to introduce RNA into a cell for the treatment or prevention of disease. For example, dsRNA may be introduced into a cancerous cell or tumor and thereby inhibit gene expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene may be selected which is required for initiation or maintenance of the disease/pathology.

WO 02/44321 PCT/US01/42682

20-04-2005 EPA 9910592

Treatment would include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

A gene derived from any pathogen may be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. The inhibitory RNA could be introduced in cells *in vitro* or *ex vivo* and then subsequently placed into an animal to affect therapy, or directly treated by *in vivo* administration. A method of gene therapy can be envisioned. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, can be envisioned.

09215257 • 1428948

The present invention could be used for treatment or development of treatments for cancers of any type, including solid tumors and leukemias, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (e.g., B cell, mixed cell, null cell, T cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast cell, and myeloid), histiocytosis malignant, Hodgkin disease, immunoproliferative small, non-Hodgkin lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, cranio-pharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adeno-

20-04-2005

Printed: 22-04-2005 TEXT 99910592

carcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, Leydig cell tumor, papilloma, Sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (e.g., Ewing, experimental, Kaposi, and mast cell), neoplasms (e.g., bone, breast, digestive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia, and for treatment of other conditions in which cells have become immortalized or transformed. The invention could be used in combination with other treatment modalities, such as chemotherapy, cryotherapy, hyperthermia, radiation therapy, and the like.

As disclosed herein, the present invention may is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, TP53, and WT1); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases,

0525257 - 171803

20-04-2005

22-04-2005 TIPA 99910592

amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

The present invention could comprise a method for producing plants with reduced susceptibility to climatic injury, susceptibility to insect damage, susceptibility to infection by a pathogen, or altered fruit ripening characteristics. The targeted gene may be an enzyme, a plant structural protein, a gene involved in pathogenesis, or an enzyme that is involved in the production of a non-proteinaceous part of the plant (i.e., a carbohydrate or lipid). If an expression construct is used to transcribe the RNA in a plant, transcription by a wound- or stress-inducible; tissue-specific (e.g., fruit, seed, anther, flower, leaf, root); or otherwise regulatable (e.g., infection, light, temperature, chemical) promoter may be used. By inhibiting enzymes at one or more points in a metabolic pathway or genes involved in pathogenesis, the effect may be enhanced: each activity will be affected and the effects may be magnified by targeting multiple different components. Metabolism may also be manipulated by inhibiting feedback control in the pathway or production of unwanted metabolic byproducts.

The present invention may be used to reduce crop destruction by other plant pathogens such as arachnids, insects, nematodes, protozoans, bacteria, or fungi. Some such plants and their pathogens are listed in *Index of Plant Diseases in the United States* (U.S. Dept. of Agriculture Handbook No. 165, 1960); *Distribution of Plant-Parasitic Nematode Species in North America* (Society of Nematologists, 1985); and *Fungi on Plants and Plant Products in the United States* (American Phytopathological Society, 1989). Insects with reduced ability to damage crops or improved ability to prevent other destructive insects from damaging crops may be produced. Furthermore, some nematodes are vectors of plant pathogens, and may be attacked by other beneficial nematodes which have no effect on plants. Inhibition of target gene activity could be used to delay or

09215257.121698

20-04-2005

Printed: 22-04-2005 EPA1 99910592

prevent entry into a particular developmental step (e.g., metamorphosis), if plant disease was associated with a particular stage of the pathogen's life cycle. Interactions between pathogens may also be modified by the invention to limit crop damage. For example, the ability of beneficial nematodes to attack their harmful prey may be enhanced by inhibition of behavior-controlling nematode genes according to the invention.

Although pathogens cause disease, some of the microbes interact with their plant host in a beneficial manner. For example, some bacteria are involved in symbiotic relationships that fix nitrogen and some fungi produce phytohormones. Such beneficial interactions may be promoted by using the present invention to inhibit target gene activity in the plant and/or the microbe.

Another utility of the present invention could be a method of identifying gene function in an organism comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceutics, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for the yeast, *D. melanogaster*, and *C. elegans* genomes, can be coupled with the invention to determine gene function in an organism (e.g., nematode). The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

WO 99/53050 PCT/US99/07865

EP14 20-04-2005

The ease with which RNA can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell/organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96-well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process. Solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The amplified RNA can be fed directly to, injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by *in vivo* or *in vitro* transcription from an expression construct used to produce the library. The construct can be replicated as individual clones of the library and transcribed to produce the RNA; each clone can then be fed to, or injected into, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example: arabidopsis, bacteria, drosophila, fungi, nematodes, viruses, zebrafish, and tissue culture cells derived from mammals.

A nematode or other organism that produces a colorimetric, fluorogenic, or luminescent signal in response to a regulated promoter (e.g., transfected with a reporter gene construct) can be assayed in an HTS format to identify DNA-binding proteins that regulate the promoter. In the assay's simplest form, inhibition of a negative regulator results in an increase of the signal and inhibition of a positive regulator results in a decrease of the signal.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the

EP02735797.1 2208

characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Pesticides may include the RNA molecule itself, an expression construct capable of expressing the RNA, or organisms transfected with the expression construct. The

WO 2004/029212 PCT/US2003/030096

pesticide of the present invention may serve as an arachnicide, insecticide, nematicide, viricide, bactericide, and/or fungicide. For example, plant parts that are accessible above ground (e.g., flowers, fruits, buds, leaves, seeds, shoots, bark, stems) may be sprayed with pesticide, the soil may be soaked with pesticide to access plant parts growing beneath ground level, or the pest may be contacted with pesticide directly. If pests interact with each other, the RNA may be transmitted between them. Alternatively, if inhibition of the target gene results in a beneficial effect on plant growth or development, the aforementioned RNA, expression construct, or transfected organism may be considered a nutritional agent. In either case, genetic engineering of the plant is not required to achieve the objectives of the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

Used as either an pesticide or nutrient, a formulation of the present invention may be delivered to the end user in dry or liquid form: for example, as a dust, granulate, emulsion, paste, solution, concentrate, suspension, or encapsulation. Instructions for safe and effective use may also be provided with the formulation. The formulation might be used directly, but concentrates would require dilution by mixing with an extender provided by the formulator or the end user. Similarly, an emulsion, paste, or suspension may require the end user to perform certain preparation steps before application. The formulation may include a combination of chemical additives known in the art such as solid carriers, minerals, solvents, dispersants, surfactants, emulsifiers, tackifiers, binders, and other adjuvants. Preservatives and stabilizers may also be added to the formulation to facilitate storage. The crop area or plant may also be treated simultaneously or separately with other pesticides or fertilizers. Methods of application include dusting, scattering or pouring, soaking, spraying, atomizing, and coating. The precise physical form and chemical composition of the formulation, and its method of application, would be chosen to promote the objectives of the invention and in accordance with prevailing circumstances. Expression constructs and transfected hosts capable of replication may also promote the persistence and/or spread of the formulation.

Description of the dsRNA Inhibition Phenomenon in *C. elegans*

The operation of the present invention was shown in the model genetic organism *Caenorhabditis elegans*.

Introduction of RNA into cells had been seen in certain biological systems to interfere with function of an endogenous gene[1,2]. Many such effects were believed to result from a simple antisense mechanism dependent on hybridization between injected single-stranded RNA and endogenous transcripts. In other cases, a more complex mechanism had been suggested. One instance of an RNA-mediated mechanism was RNA interference (RNAi) phenomenon in the nematode *C. elegans*. RNAi had been used in a variety of studies to manipulate gene expression[3,4].

Despite the usefulness of RNAi in *C. elegans*, many features had been difficult to explain. Also, the lack of a clear understanding of the critical requirements for interfering RNA led to a sporadic record of failure and partial success in attempts to extend RNAi beyond the earliest stages following injection. A statement frequently made in the literature was that sense and antisense RNA preparations are each sufficient to cause interference[3,4]. The only precedent for such a situation was in plants where the process of co-suppression had a similar history of usefulness in certain cases, failure in others, and no ability to design interference protocols with a high chance of success. Working with *C. elegans*, we discovered an RNA structure that would give effective and uniform genetic inhibition. The prior art did not teach or suggest that RNA structure was a critical feature for inhibition of gene expression. Indeed the ability of crude sense and antisense preparations to produce interference[3,4] had been taken as an indication that RNA structure was not a critical factor. Instead, the extensive plant literature and much of the ongoing research in *C. elegans* was focused on the possibility that detailed features of the target gene sequence or its chromosomal locale was the critical feature for interfering with gene expression.

The inventors carefully purified sense or antisense RNA for *unc-22* and tested each for gene-specific inhibition. While the crude sense and antisense preparations had strong interfering activity, it was found that the purified sense and antisense RNAs had only marginal inhibitory activity. This was unexpected because many techniques in molecular biology are based on the assumption that RNA produced with specific *in vitro*

PCT/US98/24551 08.12.1998

promoters (e.g., T3 or T7 RNA polymerase), or with characterized promoters *in vivo*, is produced predominantly from a single strand. The inventors had carried out purification of these crude preparations to investigate whether a small fraction of the RNA had an unusual structure which might be responsible for the observed genetic inhibition. To rigorously test whether double-stranded character might contribute to genetic inhibition, the inventors carried out additional purification of single-stranded RNAs and compared inhibitory activities of individual strands with that of the double-stranded hybrid.

The following examples are meant to be illustrative of the present invention; however, the practice of the invention is not limited or restricted in any way by them.

Analysis of RNA-Mediated Inhibition of *C. elegans* Genes

The *unc-22* gene was chosen for initial comparisons of activity as a result of previous genetic analysis that yields a semi-quantitative comparison between *unc-22* gene activity and the movement phenotypes of animals[3,8]: decreases in activity produce an increasingly severe twitching phenotype, while complete loss of function results in the additional appearance of muscle structural defects and impaired motility. *unc-22* encodes an abundant but non-essential myofilament protein[7-9]. *unc-22* mRNA is present at several thousand copies per striated muscle cell[3].

Purified antisense and sense RNAs covering a 742 nt segment of *unc-22* had only marginal inhibitory activity, requiring a very high dose of injected RNA for any observable effect (Figure 4). By contrast, a sense+antisense mixture produced a highly effective inhibition of endogenous gene activity (Figure 4). The mixture was at least two orders of magnitude more effective than either single strand in inhibiting gene expression. The lowest dose of the sense+antisense mixture tested, approximately 60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. *unc-22* expression begins in embryos with approximately 500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent inhibitory activity of the sense+antisense mixture could reflect formation of double-stranded RNA (dsRNA), or conceivably some alternate synergy between the strands. Electrophoretic analysis indicated that the injected material was predomi-

Printed: 22-04-2005 TIPA1 99910592

nantly double stranded. The dsRNA was gel purified from the annealed mixture and found to retain potent inhibitory activity. Although annealing prior to injection was compatible with inhibition, it was not necessary. Mixing of sense and antisense RNAs in low salt (under conditions of minimal dsRNA formation), or rapid sequential injection of sense and antisense strands, were sufficient to allow complete inhibition. A long interval (>1 hour) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in inhibitory activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the complementary strand.

An issue of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic response mechanism[10]. Conceivably, the inventive sense+antisense synergy could reflect a non-specific potentiation of antisense effects by such a panic mechanism. This was not found to be the case: co-injection of dsRNA segments unrelated to *unc-22* did not potentiate the ability of *unc-22* single strands to mediate inhibition. Also investigated was whether double-stranded structure could potentiate inhibitory activity when placed in *cis* to a single-stranded segment. No such potentiation was seen; unrelated double-stranded sequences located 5' or 3' of a single-stranded *unc-22* segment did not stimulate inhibition. Thus potentiation of gene-specific inhibition was observed only when dsRNA sequences exist within the region of homology with the target gene.

09215257.121808

The phenotype produced by *unc-22* dsRNA was specific. Progeny of injected animals exhibited behavior indistinguishable from characteristic *unc-22* loss of function mutants. Target-specificity of dsRNA effects using three additional genes with well characterized phenotypes (Figure 1 and Table 1). *unc-54* encodes a body wall muscle myosin heavy chain isoform required for full muscle contraction[7,11,12], *fem-1* encodes an ankyrin-repeat containing protein required in hermaphrodites for sperm production[13,14], and *hlh-1* encodes a *C. elegans* homolog of the myoD family required for proper body shape and motility[15,16]. For each of these genes, injection of dsRNA produced progeny broods exhibiting the known null mutant phenotype, while the purified single strands produced no significant reduction in gene expression. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from inhibition of the corresponding gene. The exception (segment *unc54C*, which led to an embryonic and

EPO 20-04-2005

larval arrest phenotype not seen with *unc-54* null mutants) was illustrative. This segment covers the highly conserved myosin motor domain, and might have been expected to inhibit the activity of other highly related myosin heavy chain genes[17]. This interpretation would support uses of the present invention in which nucleotide sequence comparison of dsRNA and target gene show less than 100% identity. The *unc54C* segment has been unique in our overall experience to date: effects of 18 other dsRNA segments have all been limited to those expected from characterized null mutants.

The strong phenotypes seen following dsRNA injection are indicative of inhibitory effects occurring in a high fraction of cells. The *unc-54* and *hlh-1* muscle phenotypes, in particular, are known to result from a large number of defective muscle cells[11,16]. To examine inhibitory effects of dsRNA on a cellular level, a transgenic line expressing two different GFP-derived fluorescent reporter proteins in body muscle was used. Injection of dsRNA directed to *gfp* produced dramatic decreases in the fraction of fluorescent cells (Figure 2). Both reporter proteins were absent from the negative cells, while the few positive cells generally expressed both GFP forms.

The pattern of mosaicism observed with *gfp* inhibition was not random. At low doses of dsRNA, the inventors saw frequent inhibition in the embryonically-derived muscle cells present when the animal hatched. The inhibitory effect in these differentiated cells persisted through larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically-derived striated muscles are born during early larval stages and were more resistant to inhibition. These cells have come through additional divisions (13-14 versus 8-9 for embryonic muscles[18,19]). At high concentrations of *gfp* dsRNA, inhibition was noted in virtually all striated bodywall muscles, with occasional single escaping cells including cells born in embryonic or post-embryonic stages. The nonstriated vulval muscles, born during late larval development, appeared resistant to genetic inhibition at all tested concentrations of injected RNA. The latter result is important for evaluating the use of the present invention in other systems. First, it indicates that failure in one set of cells from an organism does not necessarily indicate complete non-applicability of the invention to that organism. Second, it is important to realize that not all tissues in the organism need to be affected for the invention to be used in an organism. This may serve as an advantage in some situations.

09910592 - 22-04-2005

A few observations serve to clarify the nature of possible targets and mechanisms for RNA-mediated genetic inhibition in *C. elegans*:

First, dsRNA segments corresponding to a variety of intron and promoter sequences did not produce detectable inhibition (Table 1). Although consistent with possible inhibition at a post-transcriptional level, these experiments do not rule out inhibition at the level of the gene.

Second, dsRNA injection produced a dramatic decrease in the level of the endogenous mRNA transcript (Figure 3). Here, a *mex-3* transcript that is abundant in the gonad and early embryos[20] was targeted, where straightforward *in situ* hybridization can be performed[5]. No endogenous *mex-3* mRNA was observed in animals injected with a dsRNA segment derived from *mex-3* (Figure 3D), but injection of purified *mex-3* antisense RNA resulted in animals that retained substantial endogenous mRNA levels (Figure 3C).

Third, dsRNA-mediated inhibition showed a surprising ability to cross cellular boundaries. Injection of dsRNA for *unc-22*, *gfp*, or *lacZ* into the body cavity of the head or tail produced a specific and robust inhibition of gene expression in the progeny brood (Table 2). Inhibition was seen in the progeny of both gonad arms, ruling out a transient "nicking" of the gonad in these injections. dsRNA injected into body cavity or gonad of young adults also produced gene-specific inhibition in somatic tissues of the injected animal (Table 2).

Table 3 shows that *C. elegans* can respond in a gene-specific manner to dsRNA encountered in the environment. Bacteria are a natural food source for *C. elegans*. The bacteria are ingested, ground in the animal's pharynx, and the bacterial contents taken up in the gut. The results show that *E. coli* bacteria expressing dsRNAs can confer specific inhibitory effects on *C. elegans* nematode larvae that feed on them.

Three *C. elegans* genes were analyzed. For each gene, corresponding dsRNA was expressed in *E. coli* by inserting a segment of the coding region into a plasmid construct designed for bidirectional transcription by bacteriophage T7 RNA polymerase. The dsRNA segments used for these experiments were the same as those used in previous microinjection experiments (see Figure 1). The effects resulting from feeding these bacteria to *C. elegans* were compared to the effects achieved by microinjecting animals

WO 00/01846 PCT/US99/15357

22-04-2005 LIPA 99910592

with dsRNA.

The *C. elegans* gene *unc-22* encodes an abundant muscle filament protein. *unc-22* null mutations produce a characteristic and uniform twitching phenotype in which the animals can sustain only transient muscle contraction. When wild-type animals were fed bacteria expressing a dsRNA segment from *unc-22*, a high fraction (85%) exhibited a weak but still distinct twitching phenotype characteristic of partial loss of function for the *unc-22* gene. The *C. elegans fem-1* gene encodes a late component of the sex determination pathway. Null mutations prevent the production of sperm and lead euploid (XX) animals to develop as females, while wild type XX animals develop as hermaphrodites. When wild-type animals were fed bacteria expressing dsRNA corresponding to *fem-1*, a fraction (43%) exhibit a sperm-less (female) phenotype and were sterile. Finally, the ability to inhibit gene expression of a transgene target was assessed. When animals carrying a *gfp* transgene were fed bacteria expressing dsRNA corresponding to the *gfp* reporter, an obvious decrease in the overall level of GFP fluorescence was observed, again in approximately 12% of the population (see Figure 5, panels B and C).

09821957 • 1219908

The effects of these ingested RNAs were specific. Bacteria carrying different dsRNAs from *fem-1* and *gfp* produced no twitching, dsRNAs from *unc-22* and *fem-1* did not reduce *gfp* expression, and dsRNAs from *gfp* and *unc-22* did not produce females. These inhibitory effects were apparently mediated by dsRNA: bacteria expressing only the sense or antisense strand for either *gfp* or *unc-22* caused no evident phenotypic effects on their *C. elegans* predators.

Table 4 shows the effects of bathing *C. elegans* in a solution containing dsRNA. Larvae were bathed for 24 hours in solutions of the indicated dsRNAs (1 mg/ml), then allowed to recover in normal media and allowed to grow under standard conditions for two days. The *unc-22* dsRNA was segment ds-*unc22A* from Figure 1. *pos*-1 and *sqt*-3 dsRNAs were from the full length cDNA clones. *pos-1* encodes an essential maternally provided component required early in embyogenesis. Mutations removing *pos-1* activity have an early embryonic arrest characteristic of *skn*-like mutations[29,30]. Cloning and activity patterns for *sqt-3* have been described[31]. *C. elegans sqt-3* mutants have mutations in the *col-1* collagen gene[31]. Phenotypes of affected animals are noted. Incidences of

20-04-2005

PUBLISHED 22-04-2005 TIPA1 99910592

clear phenotypic effects in these experiments were 5-10% for *unc-22*, 50% for *pos-1*, and 5% for *sqt-3*. These are frequencies of unambiguous phenocopies; other treated animals may have had marginal defects corresponding to the target gene that were not observable. Each treatment was fully gene-specific in that *unc-22* dsRNA produced only Unc-22 phenotypes, *pos-1* dsRNA produced only Pos-1 phenotypes, and *sqt-3* dsRNA produced only Sqt-3 phenotypes.

Some of the results described herein were published after the filing of our provisional application. Those publications and a review can be cited as Fire, A., et al. Nature, 391, 806-811, 1998; Timmons, L. & Fire, A. Nature, 395, 854, 1998; and Montgomery, M.K. & Fire, A. Trends in Genetics, 14, 255-258, 1998.

The effects described herein significantly augment available tools for studying gene function in *C. elegans* and other organisms. In particular, functional analysis should now be possible for a large number of interesting coding regions[21] for which no specific function have been defined. Several of these observations show the properties of dsRNA that may affect the design of processes for inhibition of gene expression. For example, one case was observed in which a nucleotide sequence shared between several myosin genes may inhibit gene expression of several members of a related gene family.

GB0224525.7 22/04/05

Methods of RNA Synthesis and Microinjection

RNA was synthesized from phagemid clones with T3 and T7 RNA polymerase[6], followed by template removal with two sequential DNase treatments. In cases where sense, antisense, and mixed RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original "sense" and "antisense" preparations. Nonetheless, RNA species accounting for less than 10% of purified RNA preparations would not have been observed. Without gel purification, the "sense" and "antisense" preparations produced significant inhibition. This inhibitory activity was reduced or eliminated upon gel purification. By contrast, sense+antisense mixtures of gel purified and non-gel-purified RNA preparations produced identical effects.

Following a short (5 minute) treatment at 68°C to remove secondary structure, sense+antisense annealing was carried out in injection buffer[27] at 37°C for 10-30 minutes.

20-04-2005

22-04-2005 TIPAT 99910592

Formation of predominantly double stranded material was confirmed by testing migration on a standard (non-denaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for double-stranded RNA of the appropriate length. Co-incubation of the two strands in a low-salt buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of double-stranded RNA *in vitro*. Non-annealed sense+antisense RNAs for *unc22B* and *gfpG* were tested for inhibitory effect and found to be much more active than the individual single strands, but 2-4 fold less active than equivalent pre-annealed preparations.

After pre-annealing of the single strands for *unc22A*, the single electrophoretic species corresponding in size to that expected for dsRNA was purified using two rounds of gel electrophoresis. This material retained a high degree of inhibitory activity.

Except where noted, injection mixes were constructed so animals would receive an average of 0.5×10^6 to 1.0×10^6 molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections were compared with equal masses of RNA (i.e., dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible; however, such variability would not affect any of the conclusions drawn herein.

09251257.1221898

Methods for Analysis of Phenotypes

Inhibition of endogenous genes was generally assayed in a wild type genetic background (N2). Features analyzed included movement, feeding, hatching, body shape, sexual identity, and fertility. Inhibition with *gfp* [27] and *lacZ* activity was assessed using strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccIs4251) made up of three plasmids: pSAK4 (*myo-3* promoter driving mitochondrially targeted GFP), pSAK2 (*myo-3* promoter driving a nuclear targeted GFP-LacZ fusion), and a *dpy-20* subclone[26] as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing a facile distinction between

20-04-2005

cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was performed by inserting the microinjection needle into the gonadal syncitium of adults and expelling 20-100 pl of solution (see Reference 25). Body cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells was another effective means of RNA delivery, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16 hour intervals. This yields a series of semi-synchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short "clearance" interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. After the clearance period, individuals are produced which show the inhibitory phenotype. After injected animals have produced eggs for several days, gonads can in some cases "revert" to produce incompletely affected or phenotypically normal progeny.

09215257 - 121868

Additional Description of the Results

Figure 1 shows genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; sequence references are as follows: *unc-22* [9], *unc-54* [12], *fem-1* [14], and *hlh-1* [15]). These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for inhibitory effects is designated with the name of the gene followed by a single letter (e.g., *unc22C*). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.

The effects of inhibitory RNA were analyzed in individual cells (Figure 2, panels A-H). These experiments were carried out in a reporter strain (called PD4251) expressing

09/10592

two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene. ds-*unc22A* RNA was injected as a negative control. GFP expression in progeny of these injected animals was not affected. The GFP patterns of these progeny appeared identical to the parent strain, with prominent fluorescence in nuclei (the nuclear localized GFP-LacZ) and mitochondria (the mitochondrially targeted GFP): young larva (Figure 2A), adult (Figure 2B), and adult body wall at high magnification (Figure 2C).

In contrast, the progeny of animals injected with ds-*gfpG* RNA are affected (Figures 2D-F). Observable GFP fluorescence is completely absent in over 95% of the cells. Few active cells were seen in larvae (Figure 2D shows a larva with one active cell; uninjected controls show GFP activity in all 81 body wall muscle cells). Inhibition was not effective in all tissues: the entire vulval musculature expressed active GFP in an adult animal (Figure 2E). Rare GFP positive body wall muscle cells were also seen adult animals (two active cells are shown in Figure 2F). Inhibition was target specific (Figures 2G-I). Animals were injected with ds-*lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. In the animals derived from this injection, mitochondrial-targeted GFP appeared unaffected while the nuclear-targeted GFP-LacZ was absent from almost all cells (larva in Figure 2G). A typical adult lacked nuclear GFP-LacZ in almost all body-wall muscles but retained activity in vulval muscles (Figure 2H). Scale bars in Figure 2 are 20 μm.

The effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA was shown by *in situ* hybridization to embryos (Figure 3, panels A-D). The 1262 nt *mex-3* cDNA clone[20] was divided into two segments, *mex-3*A and *mex-3*B with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. *mex-3*B antisense or dsRNA was injected into the gonads of adult animals, which were maintained under standard culture conditions for 24 hours before fixation and *in situ* hybridization (see Reference 5). The *mex-3*B dsRNA produced 100% embryonic arrest, while >90% of embryos from the antisense injections hatched. Antisense probes corresponding to *mex-3*A were used to

assay distribution of the endogenous *mex-3* mRNA (dark stain). Four-cell stage embryos were assayed; similar results were observed from the 1 to 8 cell stage and in the germline of injected adults. The negative control (the absence of hybridization probe) showed a lack of staining (Figure 3A). Embryos from uninjected parents showed a normal pattern of endogenous *mex-3* RNA (Figure 3B). The observed pattern of *mex-3* RNA was as previously described in Reference 20. Injection of purified *mex-3*B antisense RNA produced at most a modest effect: the resulting embryos retained *mex-3* mRNA, although levels may have been somewhat less than wild type (Figure 3C). In contrast, no *mex-3* RNA was detected in embryos from parents injected with dsRNA corresponding to *mex-3*B (Figure 3D). The scale of Figure 3 is such that each embryo is approximately 50 μm in length.

09/12/2005 17:25:57 8604712498

Gene-specific inhibitory activity by *unc-22A* RNA was measured as a function of RNA structure and concentration (Figure 4). Purified antisense and sense RNA from *unc22A* were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (*gfpG*). Injected animals were transferred to fresh culture plates 6 hours (columns labeled 1), 15 hours (columns labeled 2), 27 hours (columns labeled 3), 41 hours (columns labeled 4), and 56 hours (columns labeled 5) after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included in the graph. The bottom-left diagram shows the genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids[8,3].

Figures 5 A-C show a process and examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. A general strategy for production of dsRNA is to clone segments of interest between flanking copies of the bacteriophage T7 promoter into a bacterial plasmid construct (Figure 5A). A bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter was used as a host. A nuclease-resistant dsRNA was detected in lysates of transfected

EFS: 22-04-2005 EFAI 09910592

bacteria. Comparable inhibition results were obtained with the two bacterial expression systems. A GFP-expressing *C. elegans* strain, PD4251 (see Figure 2), was fed on a native bacterial host. These animals show a uniformly high level of GFP fluorescence in body muscles (Figure 5B). PD4251 animals were also reared on a diet of bacteria expressing dsRNA corresponding to the coding region for *gfp*. Under the conditions of this experiment, 12% of these animals showed dramatic decreases in GFP (Figure 5C). As an alternative strategy, single copies of the T7 promoter were used to drive expression of an inverted-duplication for a segment of the target gene, either *unc-22* or *gfp*. This was comparably effective.

All references (e.g., books, articles, applications, and patents) cited in this specification are indicative of the level of skill in the art and their disclosures are incorporated herein in their entirety.

09910592 - 220405

1. Izant, J. & Weintraub, H. *Cell* 36, 1007-1015 (1984).
2. Nellen, W. & Lichtenstein, C. *TIBS* 18, 419-423 (1993).
3. Fire, A., et al. *Development* 113, 503-514 (1991).
4. Guo, S. & Kemphues, K. *Cell* 81, 611-620 (1995).
5. Seydoux, G. & Fire, A. *Development* 120, 2823-2834 (1994).
6. Ausubel, F., et al. *Current Protocols in Molecular Biology*, John Wiley N.Y. (1990).
7. Brenner, S. *Genetics* 77, 71-94 (1974).
8. Moerman, D. & Baillie, D. *Genetics* 91, 95-104 (1979).
9. Benian, G., et al. *Genetics* 134, 1097-1104 (1993).
10. Proud, C. *TIBS* 20, 241-246 (1995).
11. Epstein H., et al. *J. Mol. Biol.* 90 291-300 (1974).
12. Karn, J., et al. *Proc. Natl. Acad. Sci. (U.S.A.)* 80, 4253-4257 (1983).
13. Doniach, T. & Hodgkin J.A. *Dev. Biol.* 106, 223-235 (1984).
14. Spence, A., et al. *Cell* 60, 981-990 (1990).
15. Krause, M., et al. *Cell* 63, 907-919 (1990).
16. Chen, L., et al. *Development*, 120, 1631-1641 (1994).
17. Dibb, N. J., et al. *J. Mol. Biol.* 205, 603-613 (1989).

Printed: 22-04-2005 TIPAT 99910592

18. Sulston, J., et al. *Dev. Biol.* 100, 64-119 (1983).

19. Sulston, J. & Horvitz, H. *Dev. Biol.* 82, 41-55 (1977).

20. Draper B.W., et al. *Cell* 87, 205-216 (1996).

21. Sulston, J., et al. *Nature* 356, 37-41 (1992).

22. Matzke, M. & Matzke, A. *Plant Physiol.* 107, 679-685 (1995).

23. Ratcliff, F., et al. *Science* 276, 1558-1560 (1997).

24. Latham, K. *Trends in Genetics* 12,134-138 (1996).

25. Mello, C. & Fire, A. *Methods in Cell Biology* 48, 451-482 (1995).

26. Clark, D., et al. *Mol. Gen. Genet.* 247, 367-378 (1995).

27. Chalfie, M., et al. *Science* 263, 802-805 (1994).

28. Studier, F., et al. *Methods in Enzymology* 185, 60-89 (1990).

29. Bowerman, B., et al. *Cell* 68, 1061-1075 (1992).

30. Mello, C.C., et al. *Cell* 70, 163-176 (1992).

31. van der Keyl, H., et al. *Develop. Dynamics* 201, 86-94 (1994).

32. Goeddel, D.V. *Gene Expression Technology*, Academic Press, 1990.

33. Kriegler, M. *Gene Transfer and Expression*, Stockton Press, 1990.

34. Murray, E.J. *Gene Transfer and Expression Protocols*, Humana Press, 1991.

09215257 - 22 APR 2005

20-04-2005

Printed: 22-04-2005 TIPAT 999 0592

Table 1. Effects of sense, antisense, and mixed RNAs on progeny of injected animals.

Gene and Segment		Size	Injected RNA	F1 Phenotype
unc-22			***unc-22* null mutants: strong twitchers**[7,8]	
unc22A[a]	exon 21-22	742	sense	wild type
			antisense	wild type
			sense+antisense	strong twitchers (100%)
unc22B	exon 27	1033	sense	wild type
			antisense	wild type
			sense+antisense	strong twitchers (100%)
unc22C	exon 21-22[b]	785	sense+antisense	strong twitchers (100%)
fem-1			***fem-1* null mutants: female (no sperm)**[13]	
fem1A	exon 10[c]	531	sense	hermaphrodite (98%)
			antisense	hermaphrodite (>98%)
			sense+antisense	female (72%)
fem1B	intron 8	556	sense+antisense	hermaphrodite (>98%)
unc-54			***unc-54* null mutants: paralyzed**[7,11]	
unc54A	exon 6	576	sense	wild type (100%)
			antisense	wild type (100%)
			sense+antisense	paralyzed (100%)[d]
unc54B	exon 6	651	sense	wild type (100%)
			antisense	wild type (100%)
			sense+antisense	paralyzed (100%)[d]
unc54C	exon 1-5	1015	sense+antisense	arrested embryos and larvae (100%)
unc54D	promoter	567	sense+antisense	wild type (100%)
unc54E	intron 1	369	sense+antisense	wild type (100%)
unc54F	intron 3	386	sense+antisense	wild type (100%)

8681747 - 2525260

Printed: 22-04-2005 TIPAT 99910592

Table 1 (continued).

	Gene and Segment		Size	Injected RNA	F1 Phenotype
	hlh-1			***hlh-1* null mutants: lumpy-dumpy larvae**[16]	
	hlh1A	exons 1-6	1033	sense	wild type (<2% lpy-dpy)
				antisense	wild type (<2% lpy-dpy)
				sense+antisense	lpy-dpy larvae (>90%)[e]
	hlh1B	exons 1-2	438	sense+antisense	lpy-dpy larvae (>80%)[e]
	hlh1C	exons 4-6	299	sense+antisense	lpy-dpy larvae (>80%)[e]
	hlh1D	intron 1	697	sense+antisense	wild type (<2% lpy-dpy)
	myo-3 driven GFP transgenes [f]				
	myo-3::NLS::gfp::lacZ			**makes nuclear GFP in body muscle**	
	gfpG	exons 2-5	730	sense	nuclear GFP-LacZ pattern of parent strain
				antisense	nuclear GFP-LacZ pattern of parent strain
				sense+antisense	nuclear GFP-LacZ absent in 98% of cells
	lacZL	exon 12-14	830	sense+antisense	nuclear GFP-LacZ absent in >95% of cells
	myo-3::MtLS::gfp			**makes mitochondrial GFP in body muscle**	
	gfpG	exons 2-5	730	sense	mitochondrial GFP pattern of parent strain
				antisense	mitochondrial GFP pattern of parent strain
				sense+antisense	mitochondrial GFP absent in 98% of cells
	lacZL	exon 12-14	830	sense+antisense	mitochondrial GFP pattern of parent strain

09215257 .121898

Legend of Table 1

Each RNA was injected into 6-10 adult hermaphrodites (0.5-1x10^6 molecules into each gonad arm). After 4-6 hours (to clear pre-fertilized eggs from the uterus) injected animals were transferred and eggs collected for 20-22 hours. Progeny phenotypes were scored upon hatching and subsequently at 12-24 hour intervals.

a: To obtain a semi-quantitative assessment of the relationship between RNA dose and phenotypic response, we injected each *unc22A* RNA preparation at a series of different concentrations. At the highest dose tested (3.6x10^6 molecules per gonad), the

EP2 20-04-2005

22-04-2005 IPA 99970592

individual sense and antisense *unc22A* preparations produced some visible twitching (1% and 11% of progeny respectively). Comparable doses of ds-*unc22A* RNA produced visible twitching in all progeny, while a 120-fold lower dose of ds-*unc22A* RNA produced visible twitching in 30% of progeny.

b: *unc22C* also carries the intervening intron (43 nt).

c: *fem1A* also carries a portion (131 nt) of intron 10.

d: Animals in the first affected broods (laid at 4-24 hours after injection) showed movement defects indistinguishable from those of null mutants in *unc-54*. A variable fraction of these animals (25-75%) failed to lay eggs (another phenotype of *unc-54* null mutants), while the remainder of the paralyzed animals were egg-laying positive. This may indicate partial inhibition of *unc-54* activity in vulval muscles. Animals from later broods frequently exhibit a distinct partial loss-of-function phenotype, with contractility in a subset of body wall muscles.

e: Phenotypes of *hlh-1* inhibitory RNA include arrested embryos and partially elongated L1 larvae (the *hlh-1* null phenotype) seen in virtually all progeny from injection of ds-*hlh1A* and about half of the affected animals from ds-*hlh1B* and ds-*hlh1C*) and a set of less severe defects (seen with the remainder of the animals from ds-*hlh1B* and ds-*hlh1C*). The less severe phenotypes are characteristic of partial loss of function for *hlh-1*.

f: The host for these injections, PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ. This allows simultaneous assay for inhibition of *gfp* (loss of all fluorescence) and *lacZ* (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. ds-*gfpG* caused a loss of GFP in all but 0-3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0-5 additional bodywall muscles and in the eight vulval muscles.

09215257 '122 4 9dS

20-04-2005

Table 2. Effect of injection point on genetic inhibition in injected animals and their progeny.

dsRNA	Site of injection	Injected animal phenotype	Progeny Phenotype
None	gonad or body cavity	no twitching	no twitching
None	gonad or body cavity	strong nuclear & mitochondrial GFP	strong nuclear & mitochondrial GFP
unc22B	Gonad	weak twitchers	strong twitchers
unc22B	Body Cavity Head	weak twitchers	strong twitchers
unc22B	Body Cavity Tail	weak twitchers	strong twitchers
gfpG	Gonad	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
gfpG	Body Cavity Tail	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
lacZL	Gonad	lower nuclear GFP	rare or absent nuclear GFP
lacZL	Body Cavity Tail	lower nuclear GFP	rare or absent nuclear GFP

EPA

Table 3. *C. elegans* can respond in a gene-specific manner to environmental dsRNA.

Bacterial Food	Movement	Germline Phenotype	GFP-Transgene Expression
BL21(DE3)	0% twitch	< 1% female	< 1% faint GFP
BL21(DE3) [*fem-1* dsRNA]	0% twitch	43% female	< 1% faint GFP
BL21(DE3) [*unc22* dsRNA]	85% twitch	< 1% female	< 1% faint GFP
BL21(DE3) [*gfp* dsRNA]	0% twitch	< 1% female	12% faint GFP

Table 4. Effects of bathing *C. elegans* in a solution containing dsRNA.

dsRNA	Biological Effect
unc-22	Twitching (similar to partial loss of *unc-22* function)
pos-1	Embryonic arrest (similar to loss of *pos-1* function)
sqt-3	Shortened body (Dpy) (similar to partial loss of *sqt-3* function)

Printed: 22-04-2005 EPA1 99910592

In Table 2, gonad injections were carried out into the GFP reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ. This allowed simultaneous assay of inhibition with *gfp* (fainter overall fluorescence), *lacZ* (loss of nuclear fluorescence), and *unc-22* (twitching). Body cavity injections were carried out into the tail region, to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior region of the body cavity. An equivalent set of injections was also performed into a single gonad arm. For all sites of injection, the entire progeny brood showed phenotypes identical to those described in Table 1. This included progeny produced from both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could in part be due to the persistence of products already present in the injected adult. After ds-*unc22B* injection, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21/21) of these animals. Similar effects were seen with ds-*unc22A*. Injections of ds-*gfpG* or ds-*lacZL* produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of ds-*gfpG* and ds-*lacZL* produced no twitching, while injections of ds-*unc22A* produced no change in GFP fluorescence pattern.

0215257 • 121293

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the present invention.

1516 20-04-2005

Thu, 22 04 2004 TIP4 99916592

WE CLAIM:

1. A method to inhibit expression of a target gene in a cell comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA comprises a double-stranded structure with an identical nucleotide sequence as compared to a portion of the target gene.

2. The method of claim 1 in which the target gene is a cellular gene.

3. The method of claim 1 in which the target gene is an endogenous gene.

4. The method of claim 1 in which the target gene is a transgene.

5. The method of claim 1 in which the target gene is a viral gene.

6. The method of claim 1 in which the cell is from an animal.

7. The method of claim 1 in which the cell is from a plant.

8. The method of claim 6 in which the cell is from an invertebrate animal.

9. The method of claim 8 in which the cell is from a nematode.

10. The method of claim 1 in which the identical nucleotide sequence is at least 50 bases in length.

11. The method of claim 1 in which the target gene expression is inhibited by at least 10%.

12. The method of claim 1 in which the cell is present in an organism and inhibition of target gene expression demonstrates a loss-of function phenotype.

09215257 121198

20-04-2004

13. The method of claim 1 in which the RNA comprises one strand which is self-complementary.

14. The method of claim 1 in which the RNA comprises two separate complementary strands.

15. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation outside the cell.

16. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation inside the cell.

17. The method of claim 1 in which the cell is present in an organism, and the RNA is introduced within a body cavity of the organism and outside the cell.

18. The method of claim 1 in which the cell is present in an organism and the RNA is introduced by extracellular injection into the organism.

19. The method of claim 1 in which the cell is present in a first organism, and the RNA is introduced to the first organism by feeding a second, RNA-containing organism to the first organism.

20. The method of claim 19 in which the second organism is engineered to produce an RNA duplex.

21. The method of claim 1 in which an expression construct in the cell produces the RNA.

22. A method to inhibit expression of a target gene comprising:

(a) providing an organism containing a target cell, wherein the target cell contains the target gene and the target gene is expressed in the target cell;

09/857,138 - 05/15/2001

Printed: 22-04-2005 IPAU 9991592

(b) contacting a ribonucleic acid (RNA) with the organism, wherein the RNA is comprised of a double-stranded structure with duplexed ribonucleic acid strands and one of the strands is able to duplex with a portion of the target gene; and

(c) introducing the RNA into the target cell, thereby inhibiting expression of the target gene.

23. The method of claim 22 in which the organism is an animal.

24. The method of claim 22 in which the organism is a plant.

25. The method of claim 22 in which the organism is an invertebrate animal.

26. The method of claim 22 in which the organism is a nematode.

27. The method of claim 26 in which a formulation comprised of the RNA is applied on or adjacent to a plant, and disease associated with nematode infection of the plant is thereby reduced.

28. The method of claim 22 in which the identical nucleotide sequence is at least 50 nucleotides in length.

29. The method of claim 22 in which the expression of the target gene is inhibited by at least 10%.

30. The method of claim 22 in which the RNA is introduced within a body cavity of the organism and outside the target cell.

31. The method of claim 22 in which the RNA is introduced by extracellular injection into the organism.

09215257 - 121803

20-04-2005

32. The method of claim 22 in which the organism is contacted with the RNA by feeding the organism food containing the RNA.

33. The method of claim 32 in which a genetically-engineered host transcribing the RNA comprises the food.

34. The method of claim 22 in which at least one strand of the RNA is produced by transcription of an expression construct.

35. The method of claim 35 in which the organism is a nematode and the expression construct is contained in a plant, and disease associated with nematode infection of the plant is thereby reduced.

36. A cell containing an expression construct,

wherein the expression construct transcribes at least one ribonucleic acid (RNA) and the RNA forms a double-stranded structure with duplexed strands of ribonucleic acid,

whereby said cell contains the double-stranded RNA structure and is able to inhibit expression of a target gene when the RNA is contacted with an organism containing the target gene.

37. A transgenic animal containing said cell of claim 36.

38. A transgenic plant containing said cell of claim 36.

39. A kit comprising reagents for inhibiting expression of a target gene in a cell,

wherein said kit comprises a means for introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, and

wherein the RNA has a double-stranded structure with an identical nucleotide sequence as compared to a portion of the target gene.

09215257 - 121808

Date: 22-04-2005 TIBA1 990-0592

ABSTRACT OF THE DISCLOSURE

A process is provided of introducing an RNA into a living cell to inhibit gene expression of a target gene in that cell. The process may be practiced *ex vivo* or *in vivo*. The RNA has a region with double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequences of the duplex region of the RNA and of a portion of the target gene are identical. The present invention is distinguished from prior art interference in gene expression by antisense or triple-strand methods.

09215257 * 121898

20-04-2005

09215257 .1218981

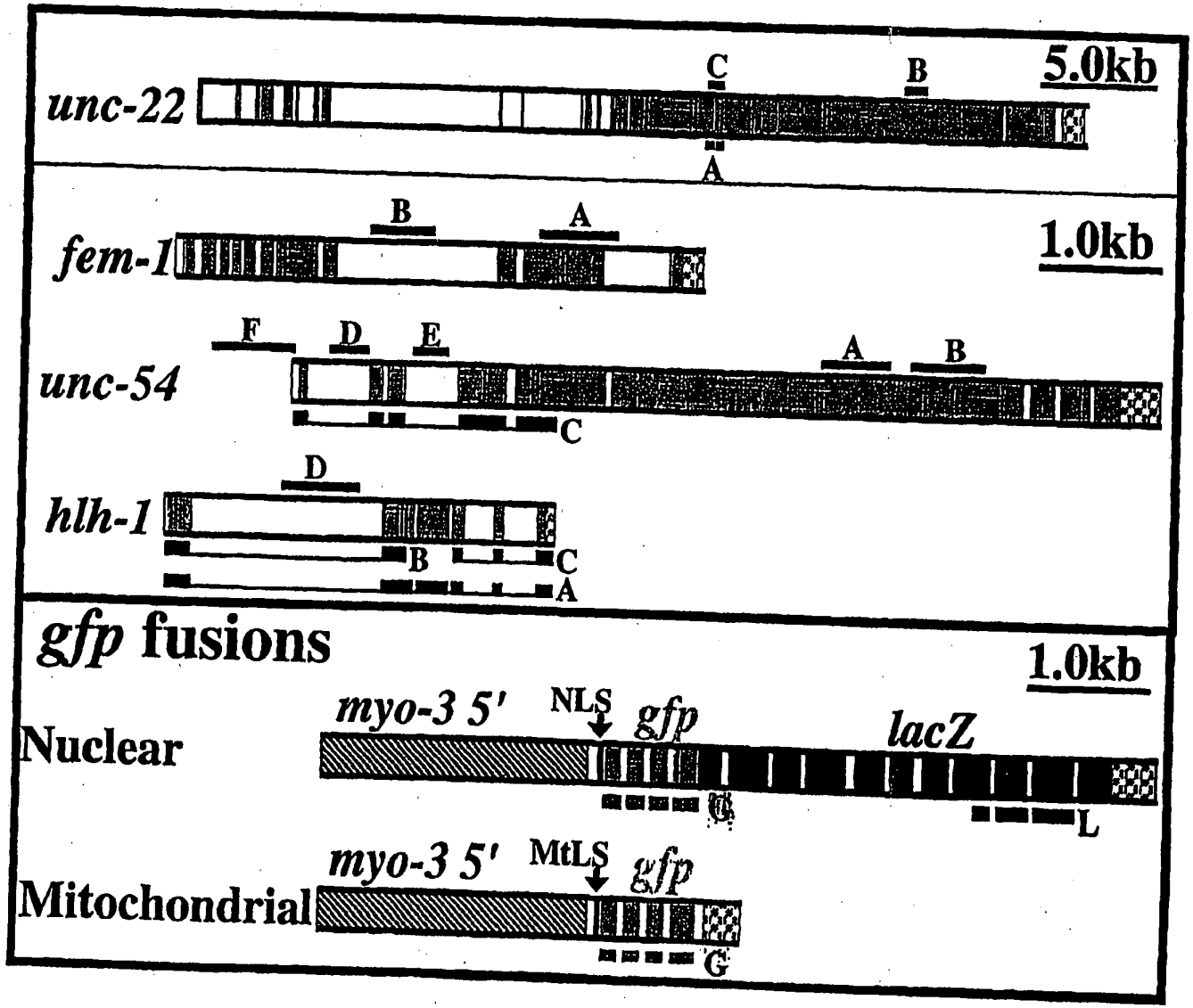


FIGURE 1

TIPA9
99913592

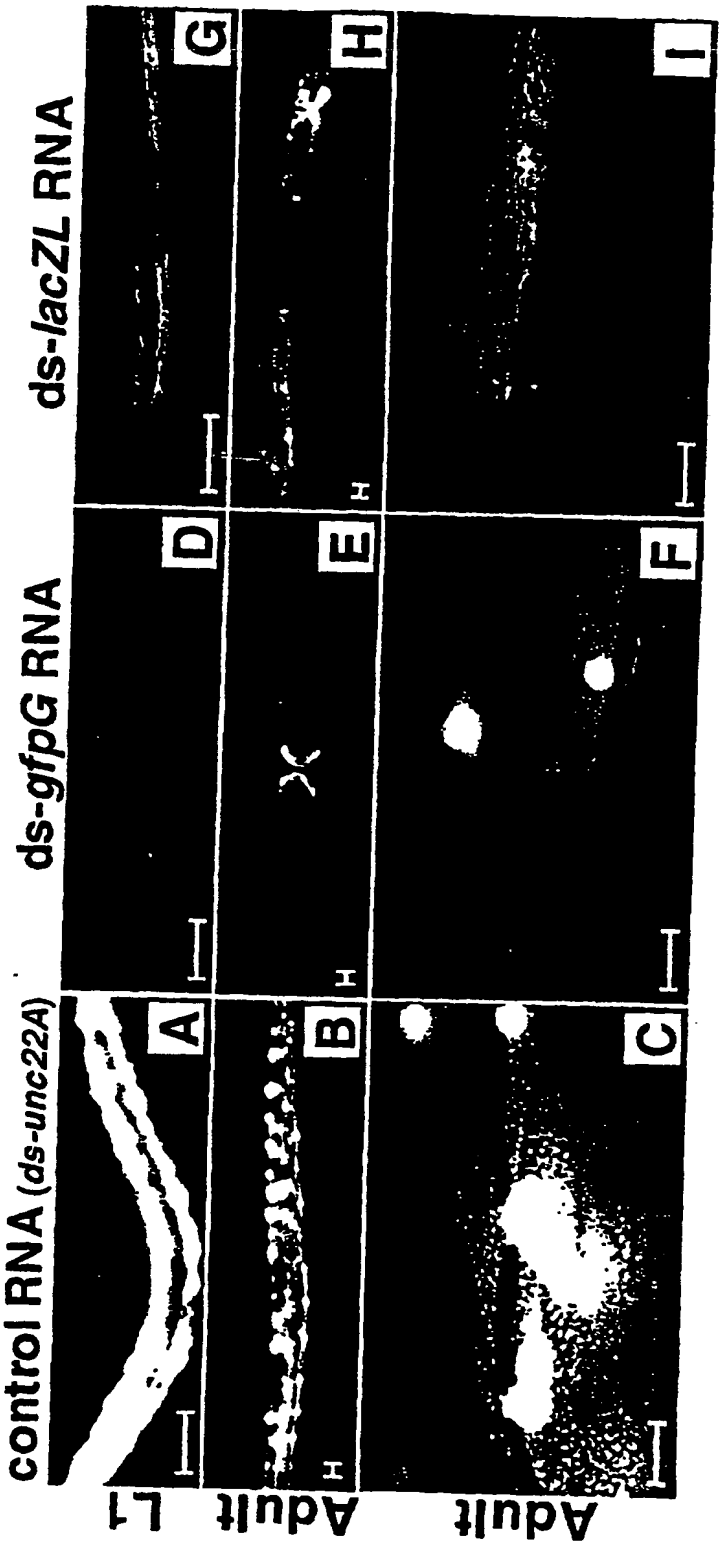


FIGURE 2

09215251260
04127.45



868121 '45251260

FIGURE 3

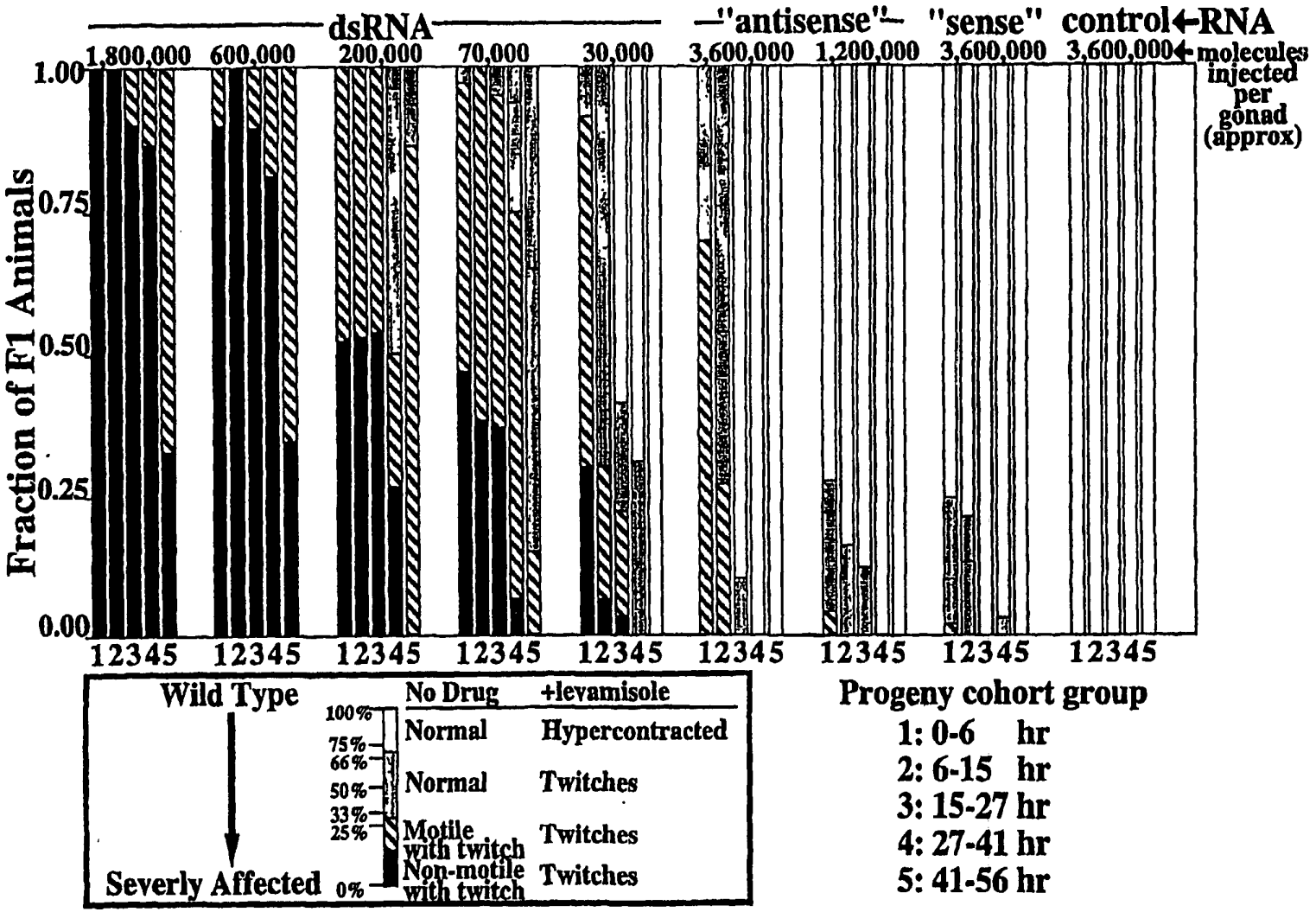

dsRNA
"antisense"
"sense" control
RNA
1,800,000
600,000
200,000
70,000
30,000
3,600,000
1,200,000
3,600,000
3,600,000
molecules injected per gonad (approx)
Fraction of F1 Animals
1.00
0.75
0.50
0.25
0.00
12345
Wild Type
Severly Affected
100%
75%
66%
50%
33%
25%
0%
No Drug
+levamisole
Normal
Hypercontracted
Normal
Twitches
Motile with twitch
Twitches
Non-motile with twitch
Twitches
Progeny cohort group
1: 0-6 hr
2: 6-15 hr
3: 15-27 hr
4: 27-41 hr
5: 41-56 hr

FIGURE 4

A
PD4251 worms
B
PD4251 worms fed bacteria expressing gfp dsRNA
C
bacterial cell wall
T7 promoter
expression plasmid
lacUV5
T7 promoter
lacI
amp
(DE3)
(*double T7*)

PIGURE 5

Printed: 22-04-2005 TIPAT 99910592

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/11

A1

(11) International Publication Number: WO 99/49029

(43) International Publication Date: 30 September 1999 (30.09.99)

(21) International Application Number: PCT/AU99/00195

(22) International Filing Date: 19 March 1999 (19.03.99)

(30) Priority Data:

PP 2492	20 March 1998 (20.03.98)	AU
PP 2499	20 March 1998 (20.03.98)	AU

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	09/100,812 (CIP)
Filed on	19 June 1998 (19.06.98)
US	09/100,813 (CIP)
Filed on	19 June 1998 (19.06.98)

(71) Applicants *(for all designated States except US)*: AG–GENE AUSTRALIA LIMITED [AU/AU]; Level 4, 62 Pitt Street, Sydney, NSW 2000 (AU). STATE OF QUEENSLAND through its DEPARTMENT OF PRIMARY INDUSTRIES [AU/AU]; Primary Industries Building, 80 Ann Street, Brisbane, QLD 4000 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: GRAHAM, Michael, Wayne [AU/AU]; 42 Raglan Street, St. Lucia, QLD 4076 (AU). RICE, Robert, Norman [AU/AU]; 39 Foley Place, Sinnamon Park, QLD 4073 (AU).

(74) Agents: HUGHES, E., John, L. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

(54) Title: CONTROL OF GENE EXPRESSION

(57) Abstract

The present invention relates generally to a method of modifying gene expression and to synthetic genes for modifying endogenous gene expression in a cell, tissue or organ of a transgenic organism, in particular a transgenic animal or plant. More particularly, the present invention utilises recombinant DNA technology to post–transcriptionally modify or modulate the expression of a target gene in a cell, tissue, organ or whole organism, thereby producing novel phenotypes. Novel synthetic genes and genetic constructs which are capable or repressing delaying or otherwise reducing the expression of an endogenous gene or a target gene in an organism when introduced thereto are also provided.

EPO - DG 1

20. 04. 2005

(93)

26-04-2005

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

Printed: 22-04-2005 AUPA 99/05952

CONTROL OF GENE EXPRESSION

FIELD OF THE INVENTION

The present invention relates generally to a method of modifying gene expression and to synthetic genes for modifying endogenous gene expression in a cell, tissue or organ of a transgenic organism, in particular a transgenic animal or plant. More particularly, the present invention utiiises recombinant DNA technology to post-transcriptionally modify or modulate the expression of a target gene in a cell, tissue, organ or whole organism, thereby producing novel phenotypes. Novel synthetic genes and genetic constructs which are capable of repressing delaying or otherwise reducing the expression of an endogenous gene or a target gene in an organism when introduced thereto are also provided.

GENERAL

Bibliographic details of the publications referred to in this specification are collected at the end of the description.

As used herein the term "derived from" shall be taken to indicate that a specified integer may be obtained from a particular specified source or species, albeit not necessarily directly from that specified source or species.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The

1745 20/04/2005

PRINTED: 22-04-2005 TIPA1 99910592

invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

Sequence identity numbers (SEQ ID NOS.) containing nucleotide and amino acid sequence information included in this specification are collected after the Abstract and have been prepared using the programme PatentIn Version 2.0. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length, type of sequence (DNA, protein (PRT), etc) and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (eg. <400>1, <400>2, etc).

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

20-04-2005

Received 22-04-2005 IPEA 99/49029

The designation of amino acid residues referred to herein, as recommended by the IUPAC-IUB Biochemical Nomenclature Commission, are listed in Table 1.

TABLE 1

Amino Acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Aspartate/Asparagine	Baa	B
Glutamate/Glutamine	Zaa	Z
Any amino acid	Xaa	X

20-04-2005

Printed: 22-04-2005 TIPAU 99910592

BACKGROUND TO THE INVENTION

Controlling metabolic pathways in eukaryotic organisms is desirable for the purposes of producing novel traits therein or introducing novel traits into a particular cell, tissue or organ of said organism. Whilst recombinant DNA technology has provided significant progress in an understanding of the mechanisms regulating eukaryotic gene expression, much less progress has been made in the actual manipulation of gene expression to produce novel traits. Moreover, there are only limited means by which human intervention may lead to a modulation of the level of eukaryotic gene expression.

One approach to repressing, delaying or otherwise reducing gene expression utilise a mRNA molecule which is transcribed from the complementary strand of a nuclear gene to that which is normally transcribed and capable of being translated into a polypeptide. Although the precise mechanism involved in this approach is not established, it has been postulated that a double-stranded mRNA may form by base pairing between the complementary nucleotide sequences, to produce a complex which is translated at low efficiency and/or degraded by intracellular ribonuclease enzymes prior to being translated.

Alternatively, the expression of an endogenous gene in a cell, tissue or organ may be suppressed when one or more copies of said gene, or one or more copies of a substantially similar gene are introduced into the cell. Whilst the mechanism involved in this phenomenon has not been established and appears to be involve mechanistically heterogeneous processes. For example, this approach has been postulated to involve transcriptional repression, in which case somatically-heritable repressed states of chromatin are formed or alternatively, a post-transcriptional silencing wherein transcription initiation occurs normally but the RNA products of the co-suppressed genes are subsequently eliminated.

The efficiency of both of these approaches in targeting the expression of specific

152 20-04-2005

IPAU 22-04-2005 IPAU 99910592

genes is very low and highly variable results are usually obtained. Inconsistent results are obtained using different regions of genes, for example 5'- untranslated regions, 3'-untranslated regions, coding regions or intron sequences to target gene expression. Accordingly, there currently exists no consensus as to the nature of genetic sequences which provide the most efficient means for repressing, delaying or otherwise reducing gene expression using existing technologies. Moreover, such a high degree of variation exists between generations such that it is not possible to predict the level of repression of a specific gene in the progeny of an organism in which gene expression was markedly modified.

Recently, Dorer and Henikoff (1994) demonstrated the silencing of tandemly repeated gene copies in the *Drosophila* genome and the transcriptional repression of dispersed *Drosophila Adh* genes by *Polycomb* genes (i.e. the *Pc-G* system; Pal-Bhadra *et al*, 1997). However, such silencing of tandemly repeated gene copies is of little utility in an attempt to manipulate gene expression in an animal cell by recombinant means, wherein the sequences capable of targeting the expression of a particular gene are introduced at dispersed locations in the genome, absent the combination of this approach with gene-targeting technology. Whilst theoretically possible, such combinations would be expected to work at only low-efficiency, based upon the low efficiency of gene-targeting approaches used in isolation and further, would require complicated vector systems. Additionally, the utilisation of transcriptional repression, such as the *Drosophila Pc-G* system, would appear to require some knowledge of the regulatory mechanisms capable of modulating the expression of any specific target gene and, as a consequence, would be difficult to implement in practice as a general technology for repressing, delaying or reducing gene expression in animal cells.

The poor understanding of the mechanisms involved in these phenomena has meant that there have been few improvements in technologies for modulating the level of gene expression , in particular technologies for delaying, repressing or otherwise reducing the expression of specific genes using recombinant DNA technology.

20-04-2005

Printed: 22-04-2005 IPA 99910592

Furthermore, as a consequence of the unpredictability of these approaches, there is currently no commercially-viable means for modulating the level of expression of a specific gene in a eukaryotic or prokaryotic organism.

Thus, there exists a need for improved methods of modulating gene expression, in particular repressing, delaying or otherwise reducing gene expression in animal cells for the purpose of introducing novel phenotypic traits thereto. In particular, these methods should provide general means for phenotypic modification, without the necessity for performing concomitant gene-targeting approaches.

SUMMARY OF THE INVENTION

The invention is based in part on the surprising discovery by the inventors that cells which exhibit one or more desired traits can be produced and selected from transformed cells comprising a nucleic acid molecule operably linked to a promoter, wherein the transcription product of the nucleic acid molecule comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of a transcript of an endogenous or non-endogenous target gene, the expression of which is intended to be modulated. The transformed cells are regenerated into whole tissues, organs or organisms capable of exhibiting novel traits, in particular virus resistance and modified expression of endogenous genes.

Accordingly, one aspect of the present invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the step of introducing to said cell, tissue or organ one or more dispersed nucleic acid molecules or foreign nucleic acid molecules comprising multiple copies of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or complementary thereto for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

154 20-04-2005

In a particularly preferred embodiment, the dispersed nucleic acid molecules or foreign nucleic acid molecules comprises a nucleotide sequence which encodes multiple copies of an mRNA molecule which is substantially identical to the nucleotide sequence of the mRNA product of the target gene. More preferably, the multiple copies of the target molecule are tandem direct repeat sequences.

In a more particularly preferred embodiment, the dispersed nucleic acid molecule or foreign nucleic acid molecule is in an expressible form such that it is at least capable of being transcribed to produce mRNA.

The target gene may be a gene which is endogenous to the animal cell or alternatively, a foreign gene such as a viral or foreign genetic sequence, amongst others. Preferably, the target gene is a viral genetic sequence.

The invention is particularly useful in the modulation of eukaryotic gene expression, in particular the modulation of human or animal gene expression and even more particularly in the modulation of expression of genes derived from vertebrate and invertebrate animals, such as insects, aquatic animals (eg. fish, shellfish, molluscs, crustaceans such as crabs, lobsters and prawns, avian animals and mammals, amongst others).

A variety of traits are selectable with appropriate procedures and sufficient numbers of transformed cells. Such traits include, but are not limited to, visible traits, disease-resistance traits, and pathogen-resistance traits. The modulatory effect is applicable to a variety of genes expressed in plants and animals including, for example, endogenous genes responsible for cellular metabolism or cellular transformation, including oncogenes, transcription factors and other genes which encode polypeptides involved in cellular metabolism.

For example, an alteration in the pigment production in mice can be engineered by

Printed: 22-04-2005 TEXT 99910592

targeting the expression of the tyrosinase gene therein. This provides a novel phenotype of albinism in black mice. By targeting genes required for virus replication in a plant cell or an animal cell, a genetic construct which comprises multiple copies of nucleotide sequence encoding a viral replicase, polymerase, coat protein or uncoating gene, or protease protein, may be introduced into a cell where it is expressed, to confer immunity against the virus upon the cell.

In performance of the present invention, the dispersed nucleic acid molecule or foreign nucleic acid molecule will generally comprise a nucleotide sequence having greater than about 85% identity to the target gene sequence, however, a higher homology might produce a more effective modulation of expression of the target gene sequence. Substantially greater homology, or more than about 90% is preferred, and even more preferably about 95% to absolute identity is desirable.

The introduced dispersed nucleic acid molecule or foreign nucleic acid molecule sequence, needing less than absolute homology, also need not be full length, relative to either the primary transcription product or fully processed mRNA of the target gene. A higher homology in a shorter than full length sequence compensates for a longer less homologous sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments will be equally effective. Normally, a sequence of greater than 20-100 nucleotides should be used, though a sequence of greater than about 200-300 nucleotides would be preferred, and a sequence of greater than 500-1000 nucleotides would be especially preferred depending on the size of the target gene.

A second aspect of the present invention provides a synthetic gene which is capable of modifying target gene expression in a cell, tissue or organ of a prokaryotic or eukaryotic organism which is transfected or transformed therewith, wherein said synthetic gene at least comprises a dispersed nucleic acid molecular foreign nucleic acid molecule comprising multiple copies of a nucleotide sequence which is

156 20-04-2005

Printed: 22-04-2005 IPAU 99910592

substantially identical to the nucleotide sequence of said target gene or a derivative thereof or a complementary sequence thereto placed operably under the control of a promoter sequence which is operable in said cell, tissue or organ.

A third aspect of the invention provides a synthetic gene which is capable of modifying the expression of a target gene in a cell, tissue or organ of a prokaryotic or eukaryotic organism which is transfected or transformed therewith, wherein said synthetic gene at least comprises multiple structural gene sequences, wherein each of said structural gene sequences comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a derivative thereof or a complementary sequence thereto and wherein said multiple structural gene sequences are placed operably under the control of a single promoter sequence which is operable in said cell, tissue or organ.

A fourth aspect of the present invention provides a synthetic gene which is capable of modifying the expression of a target gene in a cell, tissue or organ of a prokaryote or eukaryote which is transfected or transformed therewith wherein said synthetic gene at least comprises multiple structural gene sequences wherein each of said structural gene sequences is placed operably under the control of a promoter sequence which is operable in said cell, tissue or organ and wherein each of said structural gene sequences comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a derivative thereof or a complementary sequence thereto.

A fifth aspect of the present invention provides a genetic construct which is capable of modifying the expression of an endogenous gene or target gene in a transformed or transfected cell, tissue or organ wherein said genetic construct at least comprises the synthetic gene of the invention and one or more origins of replication and/or selectable marker gene sequences.

20-04-2005

In order to observe many novel traits in multicellular organisms such as plants and animals, in particular those which are tissue-specific or organ-specific or developmentally-regulated, regeneration of a transformed cell carrying the synthetic genes and genetic constructs described herein into a whole organism will be required. Those skilled in the art will be aware that this means growing a whole organism from a transformed plant cell or animal cell, a group of such cells, a tissue or organ. Standard methods for the regeneration of certain plants and animals from isolated cells and tissues are known to those skilled in the art.

Accordingly, a sixth aspect of the invention provides a cell, tissue, organ or organism comprising the synthetic genes and genetic constructs described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic representation of the plasmid pEGFP-N1 MCS.

Figure 2 is a diagrammatic representation of the plasmid pCMV.cass.

Figure 3 is a diagrammatic representation of the plasmid pCMV.SV40L.cass.

Figure 4 is a diagrammatic representation of the plasmid pCMV.SV40LR.cass.

Figure 5 is a diagrammatic representation of the plasmid pCR.Bgl-GFP-Bam.

Figure 6 is a diagrammatic representation of the plasmid pBSII(SK+).EGFP.

Figure 7 is a diagrammatic representation of the plasmid pCMV.EGFP.

Figure 8 is a diagrammatic representation of the plasmid pCR.SV40L.

Printed: 22-04-2005 TEXT 99910592

Figure 9 is a diagrammatic representation of the plasmid pCR.BEV.1.

Figure 10 is a diagrammatic representation of the plasmid pCR.BEV.2.

Figure 11 is a diagrammatic representation of the plasmid pCR.BEV.3.

Figure 12 is a diagrammatic representation of the plasmid pCMV.EGFP.BEV2.

Figure 13 is a diagrammatic representation of the plasmid pCMV.BEV.2.

Figure 14 is a diagrammatic representation of the plasmid pCMV.BEV.3.

Figure 15 is a diagrammatic representation of the plasmid pCMV.VEB.

Figure 16 is a diagrammatic representation of the plasmid pCMV.BEV.GFP.

Figure 17 is a diagrammatic representation of the plasmid pCMV.BEV.SV40L-0.

Figure 18 is a diagrammatic representation of the plasmid pCMV.0.SV40L.BEV.

Figure 19 is a diagrammatic representation of the plasmid pCMV.0.SV40L.VEB.

Figure 20 is a diagrammatic representation of the plasmid pCMV.BEVx2.

Figure 21 is a diagrammatic representation of the plasmid pCMV.BEVx3.

Figure 22 is a diagrammatic representation of the plasmid pCMV.BEVx4.

Figure 23 is a diagrammatic representation of the plasmid pCMV.BEV.SV40L.BEV.

159 20-04-2005

Figure 24 is a diagrammatic representation of the plasmid pCMV.BEV.SV40L.VEB.

Figure 25 is a diagrammatic representation of the plasmid pCMV.BEV.GFP.VEB.

Figure 26 is a diagrammatic representation of the plasmid pCMV.EGFP.BEV2.PFG.

Figure 27 is a diagrammatic representation of the plasmid pCMV.BEV.SV40LR.

Figure 28 is a diagrammatic representation of the plasmid pCDNA3.Galt.

Figure 29 is a diagrammatic representation of the plasmid pCMV.Galt.

Figure 30 is a diagrammatic representation of the plasmid pCMV.EGFP.Galt.

Figure 31 is a diagrammatic representation of the plasmid pCMV.Galt.GFP.

Figure 32 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.0.

Figure 33 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.tlaG.

Figure 34 is a diagrammatic representation of the plasmid pCMV.0.SV40L.Galt.

Figure 35 is a diagrammatic representation of the plasmid pCMV.Galtx2.

Figure 36 is a diagrammatic representation of the plasmid pCMV.Galtx4.

Figure 37 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.Galt.

Figure 38 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.tlaG.

Printed:22-04-2005 TIPAT 99910592

Figure 39 is a diagrammatic representation of the plasmid pCMV.Galt.GFP.tlaG.

Figure 40 is a diagrammatic representation of the plasmid pCMV.EGFP.Galt.PFG.

Figure 41 is a diagrammatic representation of the plasmid pCMV.Galt.SV40LR.

Figure 42 is a diagrammatic representation of the plasmid pART7.

Figure 43 is a diagrammatic representation of the plasmid pART7.35S.SCBV.cass.

Figure 44 is a diagrammatic representation of the plasmid pBC.PVY.

Figure 45 is a diagrammatic representation of the plasmid pSP72.PVY.

Figure 46 is a diagrammatic representation of the plasmid pClapBC.PVY.

Figure 47 is a diagrammatic representation of the plasmid pBC.PVYx2.

Figure 48 is a diagrammatic representation of the plasmid pSP72.PVYx2.

Figure 49 is a diagrammatic representation of the plasmid pBC.PVYx3.

Figure 50 is a diagrammatic representation of the plasmid pBC.PVYx4.

Figure 51 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.

Figure 52 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.PVY.

Figure 53 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.YVP$_{\Delta}$.

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

Figure 54 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.YVP.

Figure 55 is a diagrammatic representation of the plasmid pART27.PVY

Figure 56 is a diagrammatic representation of the plasmid pART27.35S.PVY.SCBV.O.

Figure 57 is a diagrammatic representation of the plasmid pART27.35S.O.SCBV.PVY.

Figure 58 is a diagrammatic representation of the plasmid pART27.35S.O.SCBV.YVP.

Figure 59 is a diagrammatic representation of the plasmid pART7.PVYx2.

Figure 60 is a diagrammatic representation of the plasmid pART7.PVYx3.

Figure 61 is a diagrammatic representation of the plasmid pART7.PVYx4.

Figure 62 is a diagrammatic representation of the plasmid pART7.PVY.LNYV.PVY.

Figure 63 is a diagrammatic representation of the plasmid pART7.PVY.LNYV.YVP$_{\Delta}$.

Figure 64 is a diagrammatic representation of the plasmid pART7.PVY.LNYV.YVP.

Figure 65 is a diagrammatic representation of pART7.35S.PVY.SCBV.YVP.

Figure 66 is a diagrammatic representation of pART7.35S.PVYx3.SCBV.YVPx3.

Figure 67 is a diagrammatic representation of pART7.PVYx3.LNYV.YVPx3.

Figure 68 is a diagrammatic representation of the plasmid pART7.PVYMULTI.

162 20-04-2005

AU9949029 22-04-2005 IPAU 99910592

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of modulating the expression of a target gene in a cell, tissue or organ, said method at least comprising the step of introducing to said cell, tissue or organ one or more dispersed nucleic acid molecules or foreign nucleic acid molecules comprising multiple copies of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or complementary thereto for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

By "multiple copies" is meant that two or more copies of the target gene are presented in close physical connection or juxtaposed, in the same or different orientation, on the same nucleic acid molecule, optionally separated by a stuffer fragment or intergenic region to facilitate secondary structure formation between each repeat where this is required. The stuffer fragment may comprise any combination of nucleotide or amino acid residues, carbohydrate molecules or oligosaccharide molecules or carbon atoms or a homologue, analogue or derivative thereof which is capable of being linked covalently to a nucleic acid molecule.

Preferably, embodiment, the stuffer fragment comprises a sequence of nucleotides or a homologue, analogue or derivative thereof.

More preferably, the stuffer fragment comprises a sequence of nucleotides of at least about 10-50 nucleotides in length, even more preferably at least about 50-100 nucleotides in length and still more preferably at least about 100-500 nucleotides in length.

Wherein the dispersed or foreign nucleic acid molecule comprises intron/exon splice

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

junction sequences, the stuffer fragment may serve as an intron sequence placed between the 3′-splice site of the structural gene nearer the 5′-end of the gene and the 5′- splice site of the next downstream unit thereof. Alternatively, wherein it is desirable for more than two adjacent nucleotide sequence units of the dispersed foreign nucleic acid molecule to be translated, the stuffer fragment placed there between should not include an in-frame translation stop codon, absent intron/exon splice junction sequences at both ends of the stuffer fragment or the addition of a translation start codon at the 5′ end of each unit, as will be obvious to those skilled in the art.

Preferred stuffer fragments are those which encode detectable marker proteins or biologically-active analogues and derivatives thereof, for example luciferase, β-galacturonase, β-galactosidase, chloramphenicol acetyltransferase or green fluorescent protein, amongst others. Additional stuffer fragments are not excluded.

According to this embodiment, the detectable marker or an analogue or derivative thereof serves to indicate the expression of the synthetic gene of the invention in a cell, tissue or organ by virtue of its ability to confer a specific detectable phenotype thereon, preferably a visually-detectable phenotype.

As used herein, the term "modulating" shall be taken to mean that expression of the target gene is reduced in amplitude and/or the timing of gene expression is delayed and/or the developmental or tissue-specific or cell-specific pattern of target gene expression is altered, compared to the expression of said gene in the absence of the inventive method described herein.

Whilst not limiting the scope of the invention described herein, the present invention is directed to a modulation of gene expression which comprises the repression, delay or reduction in amplitude of target gene expression in a specified cell, tissue or organ of a eukaryotic organism, in particular a plant such as a monocotyledonous or dicotyledonous plant, or a human or other animal and even more particularly a

164 20-04-2005

Printed: 22-04-2005 TIPAT 99910592

vertebrate and invertebrate animal, such as an insect, aquatic animal (eg. fish, shellfish, mollusc, crustacean such as a crab, lobster or prawn, an avian animal or a mammal, amongst others).

More preferably, target gene expression is completely inactivated by the dispersed nucleic acid molecules or foreign nucleic acid molecules which has been introduced to the cell, tissue or organ.

Whilst not being bound by any theory or mode of action, the reduced or eliminated expression of the target gene which results from the performance of the invention may be attributed to reduced or delayed translation of the mRNA transcription product of the target gene or alternatively, the prevention of translation of said mRNA, as a consequence of sequence-specific degradation of the mRNA transcript of the target gene by an endogenous host cell system.

It is particularly preferred that, for optimum results, sequence-specific degradation of the mRNA transcript of the target gene occurs either prior to the time or stage when the mRNA transcript of the target gene would normally be translated or alternatively, at the same time as the mRNA transcript of the target gene would normally be translated. Accordingly, the selection of an appropriate promoter sequence to regulate expression of the introduced dispersed nucleic acid molecule or foreign nucleic acid molecule is an important consideration to optimum performance of the invention. For this reason, strong constitutive promoters or inducible promoter systems are especially preferred for use in regulating expression of the introduced dispersed nucleic acid molecules or foreign nucleic acid molecules.

The present invention clearly encompasses reduced expression wherein reduced expression of the target gene is effected by lowered transcription, subject to the proviso that a reduction in transcription is not the sole mechanism by which this occurs and said reduction in transcription is at least accompanied by reduced translation of

20-04-2005

Printed: 22-04-2005 IPA1 99910592

the steady-state mRNA pool.

The target gene may be a genetic sequence which is endogenous to the animal cell or alternatively, a non-endogenous genetic sequence, such as a genetic sequence which is derived from a virus or other foreign pathogenic organism and is capable of entering a cell and using the cell's machinery following infection.

Wherein the target gene is a non-endogenous genetic sequence to the animal cell, it is desirable that the target gene encodes a function which is essential for replication or reproduction of the viral or other pathogen. In such embodiments, the present invention is particularly useful in the prophylactic and therapeutic treatment of viral infection of an animal cell or for conferring or stimulating resistance against said pathogen.

Preferably, the target gene comprises one or more nucleotide sequences of a viral pathogen of a plant or an animal cell, tissue or organ.

For example, in the case of animals and humans, the viral pathogen may be a retrovirus, for example a lentivirus such as the immunodeficiency viruses, a single-stranded (+) RNA virus such as bovine enterovirus (BEV) or Sinbis alphavirus. Alternatively, the target gene can comprise one or more nucleotide sequences of a viral pathogen of an animal cell, tissue or organ, such as but not limited to a double-stranded DNA virus such as bovine herpes virus or herpes simplex virus I (HSV I), amongst others.

In the case of plants, the virus pathogen is preferably a potyvirus, caulimovirus, badnavirus, geminivirus, reovirus, rhabdovirus, bunyavirus, tospovirus, tenuivirus, tombusvirus, luteovirus, sobemovirus, bromovirus, cucomovirus, ilavirus, alfamovirus, tobamovirus, tobravirus, potexvirus and clostrovirus, such as but not limited to CaMV, SCSV, PVX, PVY, PLRV, and TMV, amongst others.

166 20-04-2005

Printed:22-04-2005 IPAU 99910592

With particular regard to viral pathogens, those skilled in the art are aware that virus-encoded functions may be complemented *in trans* by polypeptides encoded by the host cell. For example, the replication of the bovine herpes virus genome in the host cell may be facilitated by host cell DNA polymerases which are capable of complementing an inactivated viral DNA polymerase gene.

Accordingly, wherein the target gene is a non-endogenous genetic sequence to the animal cell, a further alternative embodiment of the invention provides for the target gene to encode a viral or foreign polypeptide which is not capable of being complemented by a host cell function, such as a virus-specific genetic sequence. Exemplary target genes according to this embodiment of the invention include, but are not limited to genes which encode virus coat proteins, uncoating proteins and RNA-dependent DNA polymerases and RNA-dependent RNA polymerases, amongst others.

In a particularly preferred embodiment of the present invention, the target gene is the BEV RNA-dependent RNA polymerase gene or a homologue, analogue or derivative thereof or PVY Nia protease-encoding sequences.

The cell in which expression of the target gene is modified may be any cell which is derived from a multicellular plant or animal, including cell and tissue cultures thereof. Preferably, the animal cell is derived from an insect, reptile, amphibian, bird, human or other mammal. Exemplary animal cells include embryonic stem cells, cultured skin fibroblasts, neuronal cells, somatic cells, haematopoietic stem cells, T-cells and immortalised cell lines such as COS, VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI-38, baby hamster kidney (BHK) or MDBK cell lines, amongst others. Such cells and cell lines are readily available to those skilled in the art. Accordingly, the tissue or organ in which expression of the target gene is modified may be any tissue or organ comprising such animal cells.

Preferably the plant cell is derived from a monocotyledonous or dicotyledonous plant

20-04-2005

Printed 22-04-2005 TIRA1 9991059

species or a cell line derived therefrom.

As used herein, the term "dispersed nucleic acid molecule" shall be taken to refer to a nucleic acid molecule which comprises one or more multiple copies, preferably tandem direct repeats, of a nucleotide sequence which is substantially identical or complementary to the nucleotide sequence of a gene which originates from the cell, tissue or organ into which said nucleic acid molecule is introduced, wherein said nucleic acid molecule is non-endogenous in the sense that it is introduced to the cell, tissue or organ of an animal via recombinant means and will generally be present as extrachromosomal nucleic acid or alternatively, as integrated chromosomal nucleic acid which is genetically-unlinked to said gene. More particularly, the "dispersed nucleic acid molecule" will comprise chromosomal or extrachromosomal nucleic acid which is unlinked to the target gene against which it is directed in a physical map, by virtue of their not being tandemly-linked or alternatively, occupying a different chromosomal position on the same chromosome or being localised on a different chromosome or present in the cell as an episome, plasmid, cosmid or virus particle.

By "foreign nucleic acid molecule" is meant an isolated nucleic acid molecule which has one or more multiple copies, preferably tandem direct repeats, of a nucleotide sequence which originates from the genetic sequence of an organism which is different from the organism to which the foreign nucleic acid molecule is introduced. This definition encompasses a nucleic acid molecule which originates from a different individual of the same lowest taxonomic grouping (i.e. the same population) as the taxonomic grouping to which said nucleic acid molecule is introduced, as well as a nucleic acid molecule which originates from a different individual of a different taxonomic grouping as the taxonomic grouping to which said nucleic acid molecule is introduced, such as a gene derived from a viral pathogen.

Accordingly, a target gene against which a foreign nucleic acid molecule acts in the performance of the invention may be a nucleic acid molecule which has been

163 20-04-2005

Printed:22-04-2005 TIPA1 99910592

introduced from one organism to another organism using transformation or introgression technologies. Exemplary target genes according to this embodiment of the invention include the green fluorescent protein-encoding gene derived from the jellyfish *Aequoria victoria* (Prasher *et al.*,1992; International Patent Publication No. WO 95/07463), tyrosinase genes and in particular the murine tyrosinase gene (Kwon *et al.*,1988), the *Escherichia coli lacI* gene which is capable of encoding a polypeptide repressor of the *lacZ* gene, the porcine α-1,3-galactosyltransferase gene (NCBI Accession No. L36535) exemplified herein, and the PVY and BEV structural genes exemplified herein or a homologue, analogue or derivative of said genes or a complementary nucleotide sequence thereto.

The present invention is further useful for simultaneously targeting the expression of several target genes which are co-expressed in a particular cell, for example by using a dispersed nucleic acid molecule or foreign nucleic acid molecule which comprises nucleotide sequences which are substantially identical to each of said co-expressed target genes.

By "substantially identical" is meant that the introduced dispersed or foreign nucleic acid molecule of the invention and the target gene sequence are sufficiently identical at the nucleotide sequence level to permit base-pairing there between under standard intracellular conditions.

Preferably, the nucleotide sequence of each repeat in the dispersed or foreign nucleic acid molecule of the invention and the nucleotide sequence of a part of the target gene sequence are at least about 80-85% identical at the nucleotide sequence level, more preferably at least about 85-90% identical, even more preferably at least about 90-95% identical and still even more preferably at least about 95-99% or 100% identical at the nucleotide sequence level.

Notwithstanding that the present invention is not limited by the precise number of

Tag 20-04-2005

Printed: 22-04-2005 IPA1 99910592

repeated sequences in the dispersed nucleic acid molecule or the foreign nucleic acid molecule of the invention, it is to be understood that the present invention requires at least two copies of the target gene sequence to be expressed in the cell.

Preferably, the multiple copies of the target gene sequence are presented in the dispersed nucleic acid molecule or the foreign nucleic acid molecule as tandem inverted repeat sequences and/or tandem direct repeat sequences. Such configurations are exemplified by the "test plasmids" described herein that comprise Galt, BEV or PVY gene regions.

Preferably, the dispersed or foreign nucleic acid molecule which is introduced to the cell, tissue or organ comprises RNA or DNA.

Preferably, the dispersed or foreign nucleic acid molecule further comprises a nucleotide sequence or is complementary to a nucleotide sequence which is capable of encoding an amino acid sequence encoded by the target gene. Even more preferably, the nucleic acid molecule includes one or more ATG or AUG translational start codons.

Standard methods may be used to introduce the dispersed nucleic acid molecule or foreign nucleic acid molecule into the cell, tissue or organ for the purposes of modulating the expression of the target gene. For example, the nucleic acid molecule may be introduced as naked DNA or RNA, optionally encapsulated in a liposome, in a virus particle as attenuated virus or associated with a virus coat or a transport protein or inert carrier such as gold or as a recombinant viral vector or bacterial vector or as a genetic construct, amongst others.

Administration means include injection and oral ingestion (e.g. in medicated food material), amongst others.

170 20-04-2005

Printed: 22-04-2005 IPAU 99910592

The subject nucleic acid molecules may also be delivered by a live delivery system such as using a bacterial expression system optimised for their expression in bacteria which can be incorporated into gut flora. Alternatively, a viral expression system can be employed. In this regard, one form of viral expression is the administration of a live vector generally by spray, feed or water where an infecting effective amount of the live vector (e.g. virus or bacterium) is provided to the animal. Another form of viral expression system is a non-replicating virus vector which is capable of infecting a cell but not replicating therein. The non-replicating viral vector provides a means of introducing to the human or animal subject genetic material for transient expression therein. The mode of administering such a vector is the same as a live viral vector.

The carriers, excipients and/or diluents utilised in delivering the subject nucleic acid molecules to a host cell should be acceptable for human or veterinary applications. Such carriers, excipients and/or diluents are well-known to those skilled in the art. Carriers and/or diluents suitable for veterinary use include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the composition is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

In an alternative embodiment, the invention provides a method of modulating the expression of a target gene in a cell, tissue or organ, said method at least comprising the steps of:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise multiple copies of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or which is complementary thereto; and

(ii) introducing said dispersed nucleic acid molecules or foreign nucleic acid molecules to said cell, tissue or organ for a time and under conditions sufficient

20-04-2005

for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

To select appropriate nucleotide sequences for targeting expression of the target gene, several approaches may be employed. In one embodiment, multiple copies of specific regions of characterised genes may be cloned in operable connection with a suitable promoter and assayed for efficacy in reducing target gene expression. Alternatively, shotgun libraries comprising multiple copies of genetic sequences may be produced and assayed for their efficacy in reducing target gene expression. The advantage associated with the latter approach is that it is not necessary to have any prior knowledge of the significance of any particular target gene in specifying an undesirable phenotype in the cell. For example, shotgun libraries comprising virus sub-genomic fragments may be employed and tested directly for their ability to confer virus immunity on the animal host cell, without prior knowledge of the role which any virus genes play in pathogenesis of the host cell.

As used herein, the term "shotgun library" is a set of diverse nucleotide sequences wherein each member of said set is preferably contained within a suitable plasmid, cosmid, bacteriophage or virus vector molecule which is suitable for maintenance and/or replication in a cellular host. The term "shotgun library" includes a representative library, in which the extent of diversity between the nucleotide sequences is numerous such that all sequences in the genome of the organism from which said nucleotide sequences is derived are present in the "set" or alternatively, a limited library in which there is a lesser degree of diversity between said sequences. The term "shotgun library" further encompasses random nucleotide sequences, wherein the nucleotide sequence comprises viral or cellular genome fragments, amongst others obtained for example by shearing or partial digestion of genomic DNA using restriction endonucleases, amongst other approaches. A "shotgun library" further includes cells, virus particles and bacteriophage particles comprising the

IPEA/AU 22 04 2005 IPEA/AU 99910592

individual nucleotide sequences of the diverse set.

Preferred shotgun libraries according to this embodiment of the invention are "representative libraries", comprising a set of tandem repeated nucleotide sequences derived from a viral pathogen of a plant or an animal.

In a particularly preferred embodiment of the invention, the shotgun library comprises cells, virus particles or bacteriophage particles comprising a diverse set of tandem-repeated nucleotide sequences which encode a diverse set of amino acid sequences, wherein the member of said diverse set of nucleotide sequences are placed operably under the control of a promoter sequence which is capable of directing the expression of said tandem-repeated nucleotide sequence in the cell.

Accordingly, the nucleotide sequence of each unit in the tandem-repeated sequence may comprise at least about 1 to 200 nucleotides in length. The use of larger fragments, particularly employing randomly sheared nucleic acid derived from viral, plant or animal genomes, is not excluded.

The introduced nucleic acid molecule is preferably in an expressible form.

By "expressible form" is meant that the subject nucleic acid molecule is presented in an arrangement such that it may be expressed in the cell, tissue, organ or whole organism, at least at the transcriptional level (i.e. it is expressed in the animal cell to yield at least an mRNA product which is optionally translatable or translated to produce a recombinant peptide, oligopeptide or polypeptide molecule).

By way of exemplification, in order to obtain expression of the dispersed nucleic acid molecule or foreign nucleic acid molecule in the cell, tissue or organ of interest, a synthetic gene or a genetic construct comprising said synthetic gene is produced, wherein said synthetic gene comprises a nucleotide sequence as described *supra* in

20-04-2005

operable connection with a promoter sequence which is capable of regulating expression therein. Thus, the subject nucleic acid molecule will be operably connected to one or more regulatory elements sufficient for eukaryotic transcription to occur.

Accordingly, a further alternative embodiment of the invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the steps of:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise multiple copies, preferably tandem repeats, of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or which is complementary thereto;

(ii) producing a synthetic gene comprising said dispersed nucleic acid molecules or foreign nucleic acid molecules;

(iii) introducing said synthetic gene to said cell, tissue or organ; and

(iv) expressing said synthetic gene in said cell, tissue or organ for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

Reference herein to a "gene" or "genes" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5′- and 3′- untranslated sequences); and/or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5′- and 3′- untranslated sequences of the gene; and/or

(iii) a structural region corresponding to the coding regions (i.e. exons) optionally further comprising untranslated sequences and/or a heterologous promoter sequence which consists of transcriptional and/or translational regulatory regions capable of

conferring expression characteristics on said structural region.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product, in particular a sense or antisense mRNA product or a peptide, oligopeptide or polypeptide or a biologically-active protein.

The term "synthetic gene" refers to a non-naturally occurring gene as hereinbefore defined which preferably comprises at least one or more transcriptional and/or translational regulatory sequences operably linked to a structural gene sequence.

The term "structural gene" shall be taken to refer to a nucleotide sequence which is capable of being transmitted to produce mRNA and optionally, encodes a peptide, oligopeptide, polypeptide or biologically active protein molecule. Those skilled in the art will be aware that not all mRNA is capable of being translated into a peptide, oligopeptide, polypeptide or protein, for example if the mRNA lacks a functional translation start signal or alternatively, if the mRNA is antisense mRNA. The present invention clearly encompasses synthetic genes comprising nucleotide sequences which are not capable of encoding peptides, oligopeptides, polypeptides or biologically-active proteins. In particular, the present inventors have found that such synthetic genes may be advantageous in modifying target gene expression in cells, tissues or organs of a prokaryotic or eukaryotic organism.

The term "structural gene region" refers to that part of a synthetic gene which comprises a dispersed nucleic acid molecule or foreign nucleic acid molecule as described herein which is expressed in a cell, tissue or organ under the control of a promoter sequence to which it is operably connected. A structural gene region may comprise one or more dispersed nucleic acid molecules and/or foreign nucleic acid molecules operably under the control of a single promoter sequence or multiple promoter sequences. Accordingly, the structural gene region of a synthetic gene may comprise a nucleotide sequence which is capable of encoding an amino acid

Printed: 22-04-2005 TIPA1 99910592

sequence or is complementary thereto. In this regard, a structural gene region which is used in the performance of the instant invention may also comprise a nucleotide sequence which encodes an animo acid sequence yet lacks a functional translation initiation codon and/or a functional translation stop codon and, as a consequence, does not comprise a complete open reading frame. In the present context, the term "structural gene region" also extends to a non-coding nucleotide sequences, such as 5′- upstream or 3′-downstream sequences of a gene which would not normally be translated in a eukaryotic cell which expresses said gene.

Accordingly, in the context of the present invention, a structural gene region may also comprise a fusion between two or more open reading frames of the same or different genes. In such embodiments, the invention may be used to modulate the expression of one gene, by targeting different non-contiguous regions thereof or alternatively, to simultaneously modulate the expression of several different genes, including different genes of a multigene family. In the case of a fusion nucleic acid molecule which is non-endogenous to the animal cell and in particular comprises two or more nucleotide sequences derived from a viral pathogen, the fusion may provide the added advantage of conferring simultaneous immunity or protection against several pathogens, by targeting the expression of genes in said several pathogens. Alternatively or in addition, the fusion may provide more effective immunity against any pathogen by targeting the expression of more than one gene of that pathogen.

Particularly preferred structural gene regions according to this aspect of the invention are those which include at least one translatable open reading frame, more preferably further including a translational start codon located at the 5′-end of said open reading frame, albeit not necessarily at the 5′-terminus of said structural gene region. In this regard, notwithstanding that the structural gene region may comprise at least one translatable open reading frame and/or AUG or ATG translational start codon, the inclusion of such sequences in no way suggests that the present invention requires translation of the introduced nucleic acid molecule to occur in order to modulate the

176 20-04-2005

Printed: 22-04-2005 TIPAT 99910592

expression of the target gene. Whilst not being bound by any theory or mode of action, the inclusion of at least one translatable open reading frame and/or translational start codon in the subject nucleic acid molecule may serve to increase stability of the mRNA transcription product thereof, thereby improving the efficiency of the invention.

The optimum number of structural gene sequences which may be involved in the synthetic gene of the present invention will vary considerably, depending upon the length of each of said structural gene sequences, their orientation and degree of identity to each other. For example, those skilled in the art will be aware of the inherent instability of palindromic nucleotide sequences *in vivo* and the difficulties associated with constructing long synthetic genes comprising inverted repeated nucleotide sequences, because of the tendency for such sequences to recombine *in vivo*. Notwithstanding such difficulties, the optimum number of structural gene sequences to be included in the synthetic genes of the present invention may be determined empirically by those skilled in the art, without any undue experimentation and by following standard procedures such as the construction of the synthetic gene of the invention using recombinase-deficient cell lines, reducing the number of repeated sequences to a level which eliminates or minimises recombination events and by keeping the total length of the multiple structural gene sequence to an acceptable limit, preferably no more than 5-10kb, more preferably no more than 2-5kb and even more preferably no more than 0.5-2.0kb in length.

Wherein the structural gene region comprises more than one dispersed nucleic acid molecule or foreign nucleic acid molecule it shall be referred to herein as a "multiple structural gene region" or similar term. The present invention clearly extends to the use of multiple structural gene regions which preferably comprise a direct repeat sequence, inverted repeat sequence or interrupted palindrome sequence of a particular structural gene, dispersed nucleic acid molecule or foreign nucleic acid molecule, or a fragment thereof.

20-04-2005

Each dispersed or foreign nucleic acid molecule contained within the multiple structural gene unit of the subject synthetic gene may comprise a nucleotide sequence which is substantially identical to a different target gene in the same organism. Such an arrangement may be of particular utility when the synthetic gene is intended to provide protection against a pathogen in a cell, tissue or organ, in particular a viral pathogen, by modifying the expression of viral target genes. For example, the multiple structural gene may comprise nucleotide sequences (i.e. two or more dispersed or foreign nucleic acid molecules) which are substantially identical to two or more target genes selected from the list comprising DNA polymerase, RNA polymerase, Nia protease, and coat protein or other target gene which is essential for viral infectivity, replication or reproduction. However, it is preferred with this arrangement that the structural gene units are selected such that the target genes to which they are substantially identical are normally expressed at approximately the same time (or later) in an infected cell, tissue or organ as (than) the multiple structural gene of the subject synthetic gene is expressed under control of the promoter sequence. This means that the promoter controlling expression of the multiple structural gene will usually be selected to confer expression in the cell, tissue or organ over the entire life cycle of the virus when the viral target genes are expressed at different stages of infection.

As with the individual sequence units of a dispersed or foreign nucleic acid molecule, the individual units of the multiple structural gene may be spatially connected in any orientation relative to each other, for example head-to-head, head-to-tail or tail-to-tail and all such configurations are within the scope of the invention.

For expression in eukaryotic cells, the synthetic gene generally comprises, in addition to the nucleic acid molecule of the invention, a promoter and optionally other regulatory sequences designed to facilitate expression of the dispersed nucleic acid molecule or foreign nucleic acid molecule.

Reference herein to a "promoter" is to be taken in its broadest context and includes the

22-04-2005 IPAU 99910592

transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5′, of a structural gene region, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression of the sense molecule and/or to alter the spatial expression and/or temporal expression of said sense molecule. For example, regulatory elements which confer copper inducibility may be placed adjacent to a heterologous promoter sequence driving expression of a sense molecule, thereby conferring copper inducibility on the expression of said molecule.

Placing a dispersed or foreign nucleic acid molecule under the regulatory control of a promoter sequence means positioning the said molecule such that expression is controlled by the promoter sequence. Promoters are generally positioned 5′ (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous

20-04-2005

Printed: 22-04-2005 IPAU 9991059

gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in the synthetic genes of the present invention include viral, fungal, bacterial, animal and plant derived promoters capable of functioning in plant, animal, insect, fungal, yeast or bacterial cells. The promoter may regulate the expression of the structural gene component constitutively, or differentially with respect to cell, the tissue or organ in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as physiological stresses, or pathogens, or metal ions, amongst others.

Preferably, the promoter is capable of regulating expression of a nucleic acid molecule in a eukaryotic cell, tissue or organ, at least during the period of time over which the target gene is expressed therein and more preferably also immediately preceding the commencement of detectable expression of the target gene in said cell, tissue or organ.

Accordingly, strong constitutive promoters are particularly preferred for the purposes of the present invention or promoters which may be induced by virus infection or the commencement of target gene expression.

Plant-operable and animal-operable promoters are particularly preferred for use in the synthetic genes of the present invention. Examples of preferred promoters include the bacteriophage T7 promoter, bacteriophage T3 promoter, SP6 promoter, *lac* operator-promoter, *tac* promoter, SV40 late promoter, SV40 early promoter, RSV-LTR promoter, CMV IE promoter, CaMV 35S promoter, SCSV promoter, SCBV promoter and the like.

In consideration of the preferred requirement for high-level expression which coincides with expression of the target gene or precedes expression of the target gene, it is

AU00 20-04-2005

IPAU 22-04-2005
TIPA1
99910592

highly desirable that the promoter sequence is a constitutive strong promoter such as the CMV-IE promoter or the SV40 early promoter sequence, the SV40 late promoter sequence, the CaMV 35S promoter, or the SCBV promoter, amongst others. Those skilled in the art will readily be aware of additional promoter sequences other than those specifically described.

In the present context, the terms "in operable connection with" or "operably under the control" or similar shall be taken to indicate that expression of the structural gene region or multiple structural gene region is under the control of the promoter sequence with which it is spatially connected; in a cell, tissue, organ or whole organism.

In a preferred embodiment of the invention, a structural gene region (i.e. dispersed nucleic acid molecule or foreign nucleic acid molecule) or multiple structural gene region is placed operably in connection with a promoter orientation relative to the promoter sequence, such that when it is transcribed an mRNA product is synthesized which, if translated, is capable of encoding a polypeptide product of the target gene or a fragment thereof.

However, the present invention is not to be limited to the use of such an arrangement and the invention clearly extends to the use of synthetic genes and genetic constructs wherein the a structural gene region or multiple structural gene region is placed in the "antisense" orientation relative to the promoter sequence, such that at least a part of the mRNA transcription product thereof is complementary to the mRNA encoded by the target gene or a fragment thereof.

Clearly, as the dispersed nucleic acid molecule, foreign nucleic acid molecule or multiple structural gene region comprises tandem direct and/or inverted repeat sequences of the target gene, all combinations of the above-mentioned configurations are encompassed by the invention.

IPAU
20-04-2005

Printed: 22-04-2005 TIRA1 99910592

In an alternative embodiment of the invention, the structural gene region or multiple structural gene region is operably connected to both a first promoter sequence and a second promoter sequence, wherein said promoters are located at the distal and proximal ends thereof such that at least one unit of said a structural gene region or multiple structural gene region is placed in the "sense" orientation relative to the first promoter sequence and in the "antisense" orientation relative to the second promoter sequence. According to this embodiment, it is also preferred that the first and second promoters be different, to prevent competition there between for cellular transcription factors which bind thereto. The advantage of this arrangement is that the effects of transcription from the first and second promoters in reducing target gene expression in the cell may be compared to determine the optimum orientation for each nucleotide sequence tested.

The synthetic gene preferably contains additional regulatory elements for efficient transcription, for example a transcription termination sequence.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in plant cells are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants or synthesized *de novo*.

As with promoter sequences, the terminator may be any terminator sequence which is operable in the cells, tissues or organs in which it is intended to be used.

Examples of terminators particularly suitable for use in the synthetic genes of the present invention include the SV40 polyadenylation signal, the HSV TK polyadenylation signal, the CYC1 terminator, ADH terminator, SPA terminator, nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens*, the

192 20-04-2005

Printed: 22-04-2005 IPEA/AU 99910592

terminator of the Cauliflower mosaic virus (CaMV) 35S gene, the *zein* gene terminator from *Zea mays*, the Rubisco small subunit gene (SSU) gene terminator sequences, subclover stunt virus (SCSV) gene sequence terminators, any *rho*-independent *E.coli* terminator, or the *lacZ* alpha terminator, amongst others.

In a particularly preferred embodiment, the terminator is the SV40 polyadenylation signal or the HSV TK polyadenylation signal which are operable in animal cells, tissues and organs, octopine synthase (OCS) or nopaline synthase (NOS) terminator active in plant cells, tissues or organs, or the *lacZ* alpha terminator which is active in prokaryotic cells.

Those skilled in the art will be aware of additional terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

Means for introducing (i.e. transfecting or transforming) cells with the synthetic genes described herein or a genetic construct comprising same are well-known to those skilled in the art.

In a further alternative embodiment, a genetic construct is used which comprises two or more structural gene regions or multiple structural gene regions wherein each of said structural gene regions is placed operably under the control of its own promoter sequence. As with other embodiments described herein, the orientation of each structural gene region may be varied to maximise its modulatory effect on target gene expression.

According to this embodiment, the promoters controlling expression of the structural gene unit are preferably different promoter sequences, to reduce competition there between for cellular transcription factors and RNA polymerases. Preferred promoters are selected from those referred to *supra*.

20-04-2005

Printed: 22-04-2005 TIPA 99910592

Those skilled in the art will know how to modify the arrangement or configuration of the individual structural genes as described *supra* to regulate their expression from separate promoter sequences.

The synthetic genes described *supra* are capable of being modified further, for example by the inclusion of marker nucleotide sequences encoding a detectable marker enzyme or a functional analogue or derivative thereof, to facilitate detection of the synthetic gene in a cell, tissue or organ in which it is expressed. According to this embodiment, the marker nucleotide sequences will be present in a translatable format and expressed, for example as a fusion polypeptide with the translation product(s) of any one or more of the structural genes or alternatively as a non-fusion polypeptide.

Those skilled in the art will be aware of how to produce the synthetic genes described herein and of the requirements for obtaining the expression thereof, when so desired, in a specific cell or cell-type under the conditions desired. In particular, it will be known to those skilled in the art that the genetic manipulations required to perform the present invention may require the propagation of a genetic construct described herein or a derivative thereof in a prokaryotic cell such as an *E. coli* cell or a plant cell or an animal cell.

The synthetic genes of the present invention may be introduced to a suitable cell, tissue or organ without modification as linear DNA in the form of a genetic construct, optionally contained within a suitable carrier, such as a cell, virus particle or liposome, amongst others. To produce a genetic construct, the synthetic gene of the invention is inserted into a suitable vector or episome molecule, such as a bacteriophage vector, viral vector or a plasmid, cosmid or artificial chromosome vector which is capable of being maintained and/or replicated and/or expressed in the host cell, tissue or organ into which it is subsequently introduced.

Accordingly a further aspect of the invention provides a genetic construct which at

20-04-2005

Printed: 22-04-2005 IPAU 99910592

least comprises the synthetic gene according to any one or more of the embodiments described herein and one or more origins of replication and/or selectable marker gene sequences.

Genetic constructs are particularly suitable for the transformation of a eukaryotic cell to introduce novel genetic traits thereto, in addition to the provision of resistance characteristics to viral pathogens. Such additional novel traits may be introduced in a separate genetic construct or, alternatively on the same genetic construct which comprises the synthetic genes described herein. Those skilled in the art will recognise the significant advantages, in particular in terms of reduced genetic manipulations and tissue culture requirements and increased cost-effectiveness, of including genetic sequences which encode such additional traits and the synthetic genes described herein in a single genetic construct.

Usually, an origin of replication or a selectable marker gene suitable for use in bacteria is physically-separated from those genetic sequences contained in the genetic construct which are intended to be expressed or transferred to a eukaryotic cell, or integrated into the genome of a eukaryotic cell.

In a particularly preferred embodiment, the origin of replication is functional in a bacterial cell and comprises the pUC or the ColE1 origin or alternatively the origin of replication is operable in a eukaryotic cell, tissue and more preferably comprises the 2 micron (2μm) origin of replication or the SV40 origin of replication.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a genetic construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin-resistance

20-04-2005

Printed: 22-04-2005 TIPAT 9910592

gene (Amp^r), tetracycline-resistance gene (Tc^r), bacterial kanamycin-resistance gene (Kan^r), is the zeocin resistance gene (Zeocin is a drug of bleomycin family which is trademark of InVitrogen Corporation), the *AURI-C* gene which confers resistance to the antibiotic aureobasidin A, phosphinothricin-resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin-resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein-encoding gene or the luciferase gene, amongst others.

Preferably, the selectable marker gene is the *npt*II gene or Kan^r gene or green fluorescent protein (GFP)-encoding gene.

Those skilled in the art will be aware of other selectable marker genes useful in the performance of the present invention and the subject invention is not limited by the nature of the selectable marker gene.

The present invention extends to all genetic constructs essentially as described herein, which include further genetic sequences intended for the maintenance and/or replication of said genetic construct in prokaryotes or eukaryotes and/or the integration of said genetic construct or a part thereof into the genome of a eukaryotic cell or organism.

As with dispersed or foreign nucleic acid molecules, standard methods described *supra* may be used to introduce synthetic genes and genetic constructs into the cell, tissue or organ for the purposes of modulating the expression of the target gene, for example liposome-mediated transfection or transformation, transformation of cells with attenuated virus particles or bacterial cells, cell mating, transformation or transfection procedures known to those skilled in the art or described by Ausubel *et al.* (1992).

Additional means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using $CaCl_2$ and variations thereof, in particular

TSG 20-04-2005

AUPI - 22-04-2005 TIPAT 99910592

the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990) microparticle bombardment, electroporation (Fromm *et al.*, 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*, 1988; Sanford, 1988), vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as described essentially by An *et al.*(1985), Herrera-Estrella *et al.* (1983a, 1983b, 1985).

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the genetic construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

In a further embodiment of the present invention, the synthetic genes and genetic constructs described herein are adapted for integration into the genome of a cell in which it is expressed. Those skilled in the art will be aware that, in order to achieve integration of a genetic sequence or genetic construct into the genome of a host cell, certain additional genetic sequences may be required. In the case of plants, left and right border sequences from the T-DNA of the *Agrobacterium tumefaciens* Ti plasmid will generally be required.

The present invention further extends to an isolated cell, tissue or organ comprising

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

the synthetic gene described herein or a genetic construct comprising same. The present invention extends further to regenerated tissues, organs and whole organisms derived from said cells, tissues and organs and to propagules and progeny thereof.

For example, plants may be regenerated from transformed plant cells or tissues or organs on hormone-containing media and the regenerated plants may take a variety of forms, such as chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). Transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques.

The present invention is further described with reference to the following non-limiting Examples.

20-04-2005

Printed: 22-04-2005 IPAU 99910592

EXAMPLE 1

Genetic constructs comprising BEV polymerase gene sequences linked to the CMV promoter sequence and/or the SV40L promoter sequence

1. Commercial Plasmids

Plasmid pBluescript II (SK+)

Plasmid pBluescript II (SK+) is commercially available from Stratagene and comprises the LacZ promoter sequence and *lac*Z-alpha transcription terminator, with a multiple cloning site for the insertion of structural gene sequences therein. The plasmid further comprises the ColE1 and fl origins of replication and ampicillin-resistance gene.

Plasmid pSVL

Plasmid pSVL is commercially-obtainable from Pharmacia and serves as a source of the SV40 late promoter sequence. The nucleotide sequence of pSVL is also publicly available as GenBank Accession Number U13868.

Plasmid pCR2.1

Plasmid pCR2.1 is commercially available from Invitrogen and comprises the LacZ promoter sequence and *lac*Z-α transcription terminator, with a cloning site for the insertion of structural gene sequences there between. Plasmid pCR2.1 is designed to clone nucleic acid fragments by virtue of the A-overhang frequently synthesized by *Taq* polymerase during the polymerase chain reaction. PCR fragments cloned in this fashion are flanked by two EcoRI sites. The plasmid further comprises the ColE1 and f1 origins of replication and kanamycin-resistance and ampicillin-resistance genes.

Plasmid pEGFP-N1 MCS

Plasmid pEGFP-N1 MCS (Figure 1; Clontech) contains the CMV IE promoter operably connected to an open reading frame encoding a red-shifted variant of wild-type green fluorescent protein (GFP; Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Inouye and Tsuji,

20-04-2005

Printed: 22-04-2005 IPAU 99910592

1994), which has been optimised for brighter fluorescence. The specific GFP variant encoded by pEGFP-N1 MCS has been disclosed by Cormack *et al.* (1996). Plasmid pEGFP-N1 MCS contains a multiple cloning site comprising *Bgl*II and *Bam*HI sites and many other restriction endonuclease cleavage sites, located between the CMV IE promoter and the GFP open reading frame. Structural genes cloned into the multiple cloning site will be expressed at the transcriptional level if they lack a functional translation start site, however such structural gene sequences will not be expressed at the protein level (i.e. translated). Structural gene sequences inserted into the multiple cloning site which comprise a functional translation start site will be expressed as GFP fusion polypeptides if they are cloned in-frame with the GFP-encoding sequence. The plasmid further comprises an SV40 polyadenylation signal downstream of the GFP open reading frame to direct proper processing of the 3'-end of mRNA transcribed from the CMV-IE promoter sequence. The plasmid further comprises the SV40 origin of replication functional in animal cells; the neomycin-resistance gene comprising SV40 early promoter (SV40 EP in Figure 1) operably connected to the neomycin/kanamycin-resistance gene derived from Tn5 (Kan/neo in Figure 1) and the HSV thymidine kinase polyadenylation signal (HSV TK poly (A) in Figure 1), for selection of transformed cells on kamanycin, neomycin or G418; the pUC19 origin of replication which is functional in bacterial cells (pUC Ori in Figure 1); and the f1 origin of replication for single-stranded DNA production (f1 Ori in Figure 1).

2. Expression cassettes

Plasmid pCMV.cass

Plasmid pCMV.cass (Figure 2) is an expression cassette for driving expression of a structural gene sequence under control of the CMV-IE promoter sequence. Plasmid pCMV.cass was derived from pEGFP-N1 MCS by deletion of the GFP open reading frame as follows: Plasmid pEGFP-N1 MCS was digested with *Pin*AI and *Not* I, blunt-ended using *Pfu*I polymerase and then re-ligated. Structural gene sequences are cloned into pCMV.cass using the multiple cloning site, which is identical to the multiple cloning site of pEGFP-N1 MCS, except it lacks the *Pin*AI site.

20-04-2005

Printed: 22-04-2005 FIEA1 99910592

Plasmid pCMV.SV40L.cass

Plasmid pCMV.SV40L.cass (Figure 3) comprises the synthetic poly A site and the SV40 late promoter sequence from plasmid pCR.SV40L (Figure 4), sub-cloned as a *Sal* I fragment, into the *Sal* I site of plasmid pCMV.cass (Figure 2), such that the CMV-IE promoter and SV40 late promoter sequences are capable of directing transcription in the same direction. Accordingly, the synthetic poly(A) site at the 5′ end of the SV40 promoter sequence is used as a transcription terminator for structural genes expressed from the CMV IE promoter in this plasmid, which also provides for the insertion of said structural gene via the multiple cloning site present between the SV40 late promoter and the synthetic poly(A) site (Figure 5). The multiple cloning sites are located behind the CMV-IE and SV40 late promoters, including *Bam*HI and *Bgl*II sites.

Plasmid pCMV.SV40LR.cass

Plasmid pCMV.SV40LR.cass (Figure 4) comprises the SV40 late promoter sequence derived from plasmid pCR.SV40L, sub-cloned as a *Sal*I fragment into the *Sal*I site of the plasmid pCMV.cass (Figure 2), such that the CMV-IE or the SV40 late promoter may drive transcription of a structural gene or a multiple structural gene unit, in the sense or antisense orientation, as desired. A multiple cloning site is positioned between the opposing CMV- IE and SV40 late promoter sequences in this plasmid to facilitate the introduction of a structural gene sequence. In order for expression of a structural gene sequence to occur from this plasmid, it must be introduced with its own transcription termination sequence located at the 3′ end, because there are no transcription termination sequences located between the opposing CMV- IE and SV40 late promoter sequences in this plasmid. Preferably, the structural gene sequence or multiple structural gene unit which is to be introduced into pCMV.SV40LR.cass will comprise both a 5′ and a 3′ polyadenylation signal as follows:

(i) where the structural gene sequence or multiple structural gene unit is to be expressed in the sense orientation from the CMV IE promoter sequence and/or in the antisense orientation from the SV40 late promoter, the 5′ polyadenylation signal will be in the antisense orientation and the 3′

20-04-2005

Printed: 22-04-2005 TIPAT 99910592

polyadenylation signal will be in the sense orientation; and

(ii) where the structural gene sequence or multiple structural gene unit is to be expressed in the antisense orientation from the CMV IE promoter sequence and/or in the sense orientation from the SV40 late promoter, the 5′ polyadenylation signal will be in the sense orientation and the 3′ polyadenylation signal will be in the antisense orientation.

Alternatively or in addition, suitably-oriented terminator sequences may be placed at the 5′-end of the CMV and SV40L promoters, as shown in Figure 4.

Alternatively, plasmid pCMV.SV40LR.cass is further modified to produce a derivative plasmid which comprises two polyadenylation signals located between the CMV IE and SV40 late promoter sequences, in approriate orientations to facilitate expression of any structural gene located therebetween in the sense or antisense orientation from either the CMV IE promoter or the SV40 promoter sequence. The present invention clearly encompasses such derivatives.

Alternatively appropriately oriented terminators could be placed upstream of the CMV and SV40L promoters such that transcriptional termination could occur after readthrough of each of the two promoters in the antisense orientation.

3. Intermediate Constructs

Plasmid pCR.Bgl-GFP-Bam

Plasmid pCR.Bgl-GFP-Bam (Figure 5) comprises an internal region of the GFP open reading frame derived from plasmid pEGFP-N1 MCS (Figure 1) placed operably under the control of the lacZ promoter. To produce this plasmid, a region of the GFP open reading frame was amplified from pEGFP-N1 MCS using the amplification primers Bgl-GFP and GFP-Bam and cloned into plasmid pCR2.1. The internal GFP-encoding region in plasmid pCR.Bgl-GFP-Bam lacks functional translational start and stop codons.

16-2 20-04-2005

Plasmid pBSII(SK+).EGFP

Plasmid pBSII(SK+).EGFP (Figure 6) comprises the EGFP open reading frame derived from plasmid pEGFP-N1 MCS (Figure 1) placed operably under the control of the *lacZ* promoter. To produce this plasmid, the EGFP encoding region of pEGFP-N1 MCS was excised as a *Not*1/*Xho*1 fragment and cloned into the *Not*1/*Xho*1 cloning sites of plasmid pBluescript II (SK+).

Plasmid pCMV.EGFP

Plasmid pCMV.EGFP (Figure 7) is capable of expressing the EGFP structural gene under the control of the CMV-IE promoter sequence. To produce this plasmid the EGFP sequence from plasmid pBSII(SK+).EGFP was excised as *Bam*HI/*Sac*I fragment and cloned into the *Bgl*II/*Sac*I sites of plasmid pCMV.cass (Figure 2).

Plasmid pCR.SV40L

Plasmid pCR.SV40L (Figure 8) comprises the SV40 late promoter derived from plasmid pSVL (GenBank Accession No. U13868; Pharmacia), cloned into pCR2.1 (Stratagene). To produce this plasmid, the SV40 late promoter was amplified using the primers SV40-1 and SV40-2 which comprise *Sal* I cloning sites to facilitate sub-cloning of the amplified DNA fragment into pCMV.cass. The primer also contains a synthetic poly (A) site at the 5′ end, such that the amplicification product comprises the synthetic poly(A) site at the 5′ end of the SV40 promoter sequence.

Plasmid pCR.BEV.1

The BEV RNA-dependent RNA polymerase coding region was amplified as a 1,385 bp DNA fragment from a full-length cDNA clone encoding same, using primers designated BEV-1 and BEV-2, under standard amplification conditions. The amplified DNA contained a 5′-*Bgl* II restriction enzyme site, derived from the BEV-1 primer sequence and a 3′*Bam*HI restriction enzyme site, derived from the BEV-2 primer sequence. Additionally, as the BEV-1 primer sequence contains a translation start signal 5′-ATG-3′ engineered at positions 15-17, the amplified BEV polymerase

Printed: 22-04-2005 TIPAT 99910592

structural gene comprises the start site in-frame with BEV polymerase-encoding nucleotide sequences, Thus, the amplified BEV polymerase structural gene comprises the ATG start codon immediately upstream (ie. juxtaposed) to the BEV polymerase-encoding sequence. There is no translation stop codon in the amplified DNA. This plasmid is present as Figure 9.

Plasmid pCR.BEV.2

The complete BEV polymerase coding region was amplified from a full-length cDNA clone encoding same, using primers BEV-1 and BEV-3. Primer BEV-3 comprises a *Bam*HI restriction enzyme site at positions 5 to 10 inclusive and the complement of a translation stop signal at positions 11 to 13. As a consequence, an open reading frame comprising a translation start signal and translation stop signal, contained between the *Bgl* II and *Bam*HI restriction sites. The amplified fragment was cloned into pCR2.1 (Stratagene) to produce plasmid pCR2.BEV.2 (Figure 10).

Plasmid pCR.BEV.3

A non-translatable BEV polymerase structural gene was amplified from a full-length BEV polymerase cDNA clone using the amplification primers BEV-3 and BEV-4. Primer BEV-4 comprises a *Bgl*II cloning site at positions 5-10 and sequences downstream of this *Bgl*II site are homologous to nucleotide sequences of the BEV polymerase gene. There is no functional ATG start codon in the amplified DNA product of primers BEV-3 and BEV-4. The BEV polymerase is expressed as part of a polyprotein and, as a consequence, there is no ATG translation start site in this gene. The amplified DNA was cloned into plasmid pCR2.1 (Stratagene) to yield plasmid pCR.BEV.3 (Figure 11).

Plasmid pCMV.EGFP.BEV2

Plasmid pCMV.EGFP.BEV2 (Figure 12) was produced by cloning the BEV polymerase sequence from pCR.BEV.2 as a BglII/BamHI fragment into the BamHI site of pCMV.EGFP.

20-04-2005

Printed: 22-04-2005 TIPAT 99910592

4. Control Plasmids

Plasmid pCMV.BEV.2

Plasmid pCMV.BEV.2 (Figure 13) is capable of expressing the entire BEV polymerase open reading frame under the control of CMV-IE promoter sequence. To produce pCMV.BEV.2, the BEV polymerase sequence from pCR.BEV.2 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.BEV.3

Plasmid pCMV.BEV.3 (Figure 14) expresses a non-translatable BEV polymerase structural gene in the sense orientation under the control of the CMV-IE promoter sequence. To produce pCMV.BEVnt, the BEV polymerase sequence from pCR.BEV.3 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.VEB

Plasmid pCMV.VEB (Figure 15) expresses an antisense BEV polymerase mRNA under the control of the CMV-IE promoter sequence. To produce plasmid pCMV.VEB, the BEV polymerase sequence from pCR.BEV.2 was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.BEV.GFP

Plasmid pCMV.BEV.GFP (Figure 16) was constructed by cloning the GFP fragment from pCR.Bgl-GFP-Bam as a BglII/BamHI fragment into BamHI-digested pCMV.BEV.2. This plasmid serves as a control in some experiments and also as an intermediate construct.

Plasmid pCMV.BEV.SV40-L

Plasmid pCMV.BEV.SV40-L (Figure 17) comprises a translatable BEV polymerase

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

structural gene derived from plasmid pCR.BEV.2 inserted in the sense orientation between the CMV-IE promoter and the SV40 late promoter sequences of plasmid pCMV.SV40L.cass. To produce plasmid pCMV.BEV.SV40L-O, the BEV polymerase structural gene was sub-cloned as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II-digested pCMV.SV40L.cass DNA.

Plasmid pCMV.O.SV40L.BEV

Plasmid pCMV.O.SV40L.BEV (Figure 18) comprises a translatable BEV polymerase structural gene derived from plasmid pCR.BEV.2 cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences present in plasmid pCMV.SV40L.cass. To produce plasmid pCMV.O.SV40L.BEV, the BEV polymerase structural gene was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.SV40L.cass DNA.

Plasmid pCMV.O.SV40L.VEB

Plasmid pCMV.O.SV40L.VEB (Figure 19) comprises an antisense BEV polymerase structural gene derived from plasmid pCR.BEV.2 cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences present in plasmid pCMV.SV40L.cass. To produce plasmid pCMV.O.SV40L.VEB, the BEV polymerase structural gene was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.SV40L.cass DNA.

5. Test Plasmids

Plasmid pCMV.BEVx2

Plasmid pCMV.BEVx2 (Figure 20) comprises a direct repeat of a complete BEV polymerase open reading frame under the control of the CMV-IE promoter sequence. In eukaryotic cells at least, the open reading frame located nearer the CMV-IE promoter is translatable. To produce pCMV.BEVx2, the BEV polymerase structural gene from plasmid pCR.BEV.2 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.2, immediately downstream of the

20-04-2005

Printed: 22-04-2005 IPAU 999 10592

BEV polymerase structural gene already present therein.

Plasmid pCMV.BEVx3

Plasmid pCMV.BEVx3 (Figure 21) comprises a direct repeat of three complete BEV polymerase open reading frames under the control of the CMV-1E promoter. To produce pCMV.BEVx3, the BEV polymerase fragment from pCR.BEV.2 was cloned in the sense orientation as a BglII/BamHI fragment into the BamHI site of pCMV.BEVx2, immediately downstream of the BEV polymerase sequences already present therein.

Plasmid pCMV.BEVx4

Plasmid pCMV.BEVx4 (Figure 22) comprises a direct repeat of four complete BEV polymerase open reading frames under the control of the CMV-1E promoter. To produce pCMV.BEVx4, the BEV polymerase fragment from pCR.BEV.2 was cloned in the sense orientation as a BglII/BamHI fragment into the BamHI site of pCMV.BEVx3, immediately downstream of the BEV polymerase sequences already present therein.

Plasmid pCMV.BEV.SV40L.BEV

Plasmid pCMV.BEV.SV40L.BEV(Figure 23) comprises a multiple structural gene unit comprising two BEV polymerase structural genes placed operably and separately under control of the CMV-IE promoter and SV40 late promoter sequences. To produce plasmid pCMV.BEV.SV40L.BEV, the translatable BEV polymerase structural gene present in pCR.BEV.2 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment behind the SV40 late promoter sequence present in *Bam*HI-digested pCMV.BEV.SV40L-O.

Plasmid pCMV.BEV.SV40L.VEB

Plasmid pCMV.BEV.SV40L.VEB (Figure 24) comprises a multiple structural gene unit comprising two BEV polymerase structural genes placed operably and separately

20-04-2005

under control of the CMV-IE promoter and SV40 late promoter sequences. To produce plasmid pCMV.BEV.SV40L.VEB, the translatable BEV polymerase structural gene present in pCR.BEV.2 was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment behind the SV40 late promoter sequence present in *Bam*HI-digested pCMV.BEV.SV40L-O. In this plasmid, the BEV polymerase structural gene is expressed in the sense orientation under control of the CMV-IE promoter to produce a translatable mRNA, whilst the BEV polymerase structural gene is also expressed under control of the SV40 promoter to produce an antisense mRNA species.

Plasmid pCMV.BEV.GFP.VEB

Plasmid pCMV.BEV.GFP.VEB (Figure 25) comprises a BEV structural gene inverted repeat or palindrome, interrupted by the insertion of a GFP open reading frame (stuffer fragment) between each BEV structural gene sequence in the inverted repeat. To produce plasmid pCMV.BEV.GFP.VEB, the GFP stuffer fragment from pCR.Bgl-GFP-Bam was first sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.2 to produce an intermediate plasmid pCMV.BEV.GFP wherein the BEV polymerase-encoding and GFP-encoding sequences are contained within the same 5′-*Bgl*II-to-*Bam*HI-3′ fragment. The BEV polymerase structural gene from pCMV.BEV.2 was then cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.GFP. The BEV polymerase structural gene nearer the CMV-IE promoter sequence in plasmid pCMV.BEV.GFP.VEB is capable of being translated, at least in eukaryotic cells.

Plasmid pCMV.EGFP.BEV2.PFG

Plasmid pCMV.EGFP.BEV2.PFG (Figure 26) comprise a GFP palindrome, interrupted by the insertion of a BEV polymerase sequence between each GFP structural gene in the inverted repeat. To produce this plasmid the GFP fragment from pCR.Bgl-GFP-Bam was cloned as a BglII/BamHI fragment into the BamHI site of pCMV.EGFP.BEV2 in the antisense orientation relative to the CMV promoter.

Plasmid pCMV.BEV.SV40LR

Plasmid pCMV.BEV.SV40LR (Figure 27) comprises a structural gene comprising the entire BEV polymerase open reading frame placed operably and separately under control of opposing CMV-IE promoter and SV40 late promoter sequences, thereby potentially producing BEV polymerase transcripts at least from both strands of the full-length BEV polymerase structural gene. To produce plasmid pCMV.BEV.SV40LR, the translatable BEV polymerase structural gene present in pCR.BEV.2 was sub-cloned, as a *Bgl*II-to-*Bam*HI fragment, into the unique *Bgl*II site of plasmid pCMV.SV40LR.cass, such that the BEV open reading frame is present in the sense orientation relative to the CMV-IE promoter sequence.

Those skilled in the art will recognise that it is possible to generate a plasmid wherein the BEV polymerase fragment from pCR.BEV.2 is inserted in the antisense orientation, relative to the CMV IE promoter sequence, using this cloning strategy. The present invention further encompasses such a genetic construct.

EXAMPLE 2

Genetic constructs comprising the porcine α-1,3-galactosyltransferase (Galt) structural gene sequence or sequences operably connected to the CMV promoter sequence and/or the SV40L promoter sequence

1. Commercial Plasmids

Plasmid pcDNA3

Plasmid pcDNA3 is commercially available from Invitrogen and comprises the CMV-IE promoter and BGHpA transcription terminator, with multiple cloning sites for the insertion of structural gene sequences there between. The plasmid further comprises the ColE1 and f1 origins of replication and neomycin-resistance and ampicillin-resistance genes.

Printed: 22-04-2005 TIPAT 999 0592

2. Intermediate plasmids

Plasmid pcDNA3.Galt

Plasmid pcDNA3.Galt (BresaGen Limited, South Australia, Australia; Figure 28) is plasmid pcDNA3 (Invitrogen) and comprises the cDNA sequence encoding porcine gene alpha-1,3-galactosyltransferase (Galt) operably under the control of the CMV-IE promoter sequence such that it is capable of being expressed therefrom. To produce plasmid pcDNA3.Galt, the porcine gene alpha-1,3-galactosyltransferase cDNA was cloned as an *Eco*RI fragment into the *Eco*RI cloning site of pcDNA3. The plasmid further comprises the ColE1 and f1 origins of replication and the neomycin and ampicillin-resistance genes.

3. Control Plasmids

Plasmid pCMV.Galt

Plasmid pCMV.Galt (Figure 29) is capable of expressing the Galt structural gene under the control of the CMV-IE promoter sequence. To produce plasmid pCMV.Galt, the Galt sequence from plasmid pcDNA3.Galt was excised as an *Eco*RI fragment and cloned in the sense orientation into the *Eco*RI site of plasmid pCMV.cass (Figure 2).

Plasmid pCMV.EGFP.Galt

Plasmid pCMV.EGFP.Galt (Figure 30) is capable of expressing the Galt structural gene as a Galt fusion polypeptide under the control of the CMV-IE promoter sequence. To produce plasmid pCMV.EGFP.Galt, the Galt sequence from pCMV.Galt (Figure 29) was excised as a *Bgl*II/*Bam*HI fragment and cloned into the *Bam*HI site of pCMV.EGFP.

Plasmid pCMV.Galt.GFP

Plasmid pCMV.Galt.GFP (Figure 31) was produced by cloning the Galt cDNA as an EcORI fragment from pCDNA3 into EcoRI-digested pCMV.EGFP in the sense orientation. This plasmid serves as both a control and construct intermediate.

20-01-2005

Plasmid pCMV.Galt.SV40L.0

The plasmid pCMV.Galt.SV40L.0 (Figure 32) comprises a Galt structural gene cloned downstream of the CMV promoter present in pCMV.SV40L.cass. To produce the plasmid the Galt cDNA fragment from pCMV.Galt was cloned as a BglII/BamHI into BglII-digested pCMV.SV40L.cass in the sense orientation.

Plasmid pCMV.O.SV40L.tlaG

The plasmid pCMV.O.SV40L.tlaG (Figure 33) comprises a Galt structural gene clones in an antisense orientation downstream of the SV40L promoter present in pCMV.SV40L.cass. To produce this plasmid the Galt cDNA fragment from pCMV.Galt was cloned as a BglII/BamHI into BamHI-digested pCMV.SV40L.cass in the antisense orientation.

Plasmid pCMV.O.SV40L.Galt

The plasmid pCMV.O.SV40L.Galt (Figure 34) comprises a Galt structural gene cloned downstream of the SV40L promoter present in pCMV.SV40L.cass. To produce the plasmid the Galt cDNA fragment from pCMV.Galt was cloned as a BglII/BamHI into BamHI-digested pCMV.SV40L.cass in the sense orientation.

4. Test Plasmids

Plasmid pCMV.Galtx2

Plasmid pCMV.Galtx2 (Figure 35) comprises a direct repeat of a Galt open reading frame under the control of the CMV-IE promoter sequence. In eukaryotes cells at least, the open reading frame located nearer the CMV-IE promoter is translatable. To produce pCMV.Galtx2, the Galt structural gene from pCMV.Galt was excised as a *Bgl*II/*Bam*HI fragment and cloned in the sense orientation into the *Bam*HI cloning site of pCMV.Galt.

Plasmid pCMV.Galtx4

Plasmid pCMV.Galtx4 (Figure 36) comprises a quadruple direct repeat of a Galt open

Printed: 22-04-2005 IPAU 99910592

reading frame under the control of the CMV-IE promoter sequence. In eukaryotes cells at least, the open reading frame located nearer the CMV-IE promoter is translatable. To produce pCMV.Galtx4, the Galtx2 sequence from pCMV.Galtx2 was excised as a *Bgl*II/*Bam*HI fragment and cloned in the sense orientation into the *Bam*HI cloning site of pCMV.Galtx2.

Plasmid pCMV.Galt.SV40L.Galt

The plasmid pCMV.Galt.SV40L.Galt (Figure 37) is designed to express two sense transcripts of Galt, one driven by the CMV promoter, the other by the SV40L promoter. To produce the plasmid a Galt cDNA fragment from pCMV.Galt was cloned as a BglII/BamHI fragment into BglII-digested pCMV.O.SV40.Galt in the sense orientation.

Plasmid pCMV.Galt.SV40L.tlaG

The plasmid pCMV.Galt.SV40.tlaG (Figure 38) is designed to express a sense transcript of Galt driven by the CMV promoter and an antisense transcript driven by the SV40L promoter. To produce the plasmid a Galt cDNA fragment from pCMV.Galt was cloned as a BglII/BamHI fragment into BglII-digested pCMV.O.SV40.talG in the sense orientation.

Plasmid pCMV.Galt.GFP.tlaG

Plasmid pCMV.Galt.GFP.tlaG (Figure 39) comprise a Galt palindrome, interrupted by the insertion of a GFP sequence between each Galt structural gene in the inverted repeat. To produce this plasmid the BglII/BamHI Galt cDNA fragment from pCMV.Galt was cloned into the BamHI site of pCMV.Galt.GFP in the antisense relative to the CMV promoter.

Plasmid pCMV.EGFP.Galt.PFG

The plasmid pCMV.EGFP.Galt.PFG (Figure 40) comprises a GFP palindrome, interrupted by the insertion of a Galt sequence between each GFP structural gene of the inverted repeat, expression of which is driven by the CMV promoter. To produce

202 20-04-2005

Printed: 22-04-2005 TIPA1 99910592

this plasmid the Galt sequences from pCMV.Galt were cloned as a BglII/BamHI fragment into BamHI-digested pCMV.EGFP in the sense orientation to produce the intermediate pCMV.EGFP.Galt (not shown); following this further GFP sequences from pCR.Bgl-pCMV.EGFP.Galt in the antisense orientation.

Plasmid pCMV.Galt.SV40LR

The plasmid pCMV.Galt.SV40LR (Figure 41) is designed to express GalT cDNA sequences cloned between the opposing CMV and SV40L promoters in the expression cassette pCMV.SV40LR.cass. To produce this plasmid Galt sequences from pCMV.Galt were cloned as a BglII/BamHI fragment in BglII-digested pCMV.SV40LR.cass in the sense orientation relative to the 35S promoter.

EXAMPLE 3

Genetic constructs comprising PVY Nia sequences operably linked to the35S promoter sequence and/or the SCBV promoter sequence

1: Binary vector

Plasmid pART27

Plasmid pART27 is a binary vector, specifically designed to be compatible with the pART7 expression cassette. It contains bacterial origins of replication for both E. coli and Agrobacterium tumefaciens, a spectinomycin resistance gene for bacterial selection, left and right T-DNA borders for transfer of DNA from Agrobacterium to plant cells and a kanamycin resistance cassette to permit selection of transformed plant cells. The kanamycin resistance cassette is located between the T-DNA borders, pART27 also contains a unique NotI restriction site which permits cloning of constructs prepared in vectors such as pART7 to be cloned between the T-DNA borders. Construction of pART27 is described in Gleave, AP (1992).

When cloning NotI inserts into this vector, two insert orientations can be obtained. In all the following examples the same insert orientation, relative to the direction of the

20-04-2005

35S promoter in the described pART7 constructs was chosen; this was done to minimise any experimental artefacts that may arise from comparing different constructs with different insert orientations.

2. Commercial plasmids

Plasmid pBC (KS-)

Plasmid pBC (KS-) is commercially available from Stratagene and comprises the LacZ promoter sequence and lacZ-alpha transcription terminator, with a multiple cloning site for the insertion of structural gene sequences therein. The plasmid further comprises the ColE1 and fl origins of replication and a chloroamphenicol-resistance gene.

Plasmid pSP72

Plasmid pSP72 is commercially available from Promega and contains a multiple cloning site for the insertion of structural gene sequences therein. The plasmid further comprises the ColE1 origin of replication and an ampicillin-resistance gene.

3. Expression cassettes

Plasmid pART7

Plasmid pART7 is an expression cassette designed to drive expression of sequences cloned behind the 35S promoter. It contains a polylinker to assist cloning and a region of the octipine synthase terminator. The 35S expression cassette is flanked by two Not I restriction sites which permits cloning into binary expression vectors, such as pART27 which contains a unique NotI site. Its construction as described in Gleave, AP (1992), a map is shown in Figure 43.

Plasmid pART7.35S.SCBV.cass

Plasmid p35S.CMV.cass was designed to express two separate gene sequences cloned into a single plasmid. To create this plasmid, sequences corresponding to the nos terminator and the SCBV promoter were amplified by PCR then cloned in the polylinker of pART7 between the 35S promoter and OCS.

The resulting plasmid has the following arrangement of elements:

35S promoter - polylinker 1 - NOS terminator - SCBV promoter - polylinker 2 - OCS terminator.

Expression of sequences cloned into polylinker 1 is controlled by the 35S promoter, expression of sequences cloned into polylinker 2 is controlled by the SCBV promoter.

The NOS terminator sequences were amplified from the plasmid pAHC27 (Christensen and Quail, 1996) using the two oligonucleotides;

NOS 5′ (forward primer; SEQ ID ??)
5′-GGATTCCCGGGACGTCGCGAATTTCCCCCGATCGTTC-3′; and

NOS 3′ (reverse primer; SEQ ID ??)
5′-CCATGGCCATATAGGCCCGATCTAGTAACATAG-3′

Nucleotide residues 1 to 17 for NOS 5′ and 1 to 15 for NOS 3′ represent additional nucleotides designed to assist in construct preparation by adding additional restriction sites. For NOS 5′ these are BamHI, SmaI, AatII and the first 4 bases of an NruI site, for NOS 3′ these are NcoI and SfiI sites. The remaining sequences for each oligonucleotide are homologous to the 5′ and 3′ ends respectively of NOS sequences in pAHC 27.

The SCBV promoter sequences were amplified from the plasmid pScBV-20 (Tzafir *et al*, 1998) using the two oligonucleotides:

SCBV 5′: 5′-CCATGGCCTATATGGCCATTCCCCACATTCAAG-3′; and

SCBV 3′: 5′-AACGTTAACTTCTACCCAGTTCCAGAG-3′

Nucleotide residues 1 to 17 of SCBV 5′ encode NcoI and SfiI restriction sites designed to assist in construct preparation, the remaining sequences are homologous to upstream sequences of the SCMV promoter region. Nucleotide residues 1 to 9 of SCBV 3′ encode Psp10461 and HpaI restriction sites designed to assist in construct preparation, the remaining sequences are homologous to the reverse and complement of sequences near the transcription initiation site of SCBV.

Sequences amplified from pScBV-20 using PCR and cloned into pCR2.1 (Invitrogen) to produce pCR.NOS and pCR.SCBV respectively. SmaI I/SfiI cut pCR.NOS and SfiI/HpaI cut pCR.SCBV were ligated into SmaI cut pART7 and a plasmid with a suitable orientation was chosen and designated pART7.35S.SCBV.cass, a map of this construct is shown in Figure 43.

4. Intermediate constructs

Plasmid pBC.PVY

A region of the PVY genome was amplified by PCR using reverse-transcribed RNA isolated from PVY-infected tobacco as a template using standard protocols and cloned into a plasmid pGEM 3 (Stratagene), to create pGEM.PVY. A SalI/HindIII fragment from pGEM.PVY, corresponding to a SalI/HindIII fragment positions 1536-2270 of the PVY strain O sequence (Acc. No D12539, Genbank), was then subcloned into the plasmid pBC (Stratagene Inc.) to create pBC.PVY (Figure 44).

Plasmid pSP72.PVY

Plasmid pSP72.PVY was prepared by inserting an EcoRI/SalI fragment from pBC.PVY into EcoRI/SalI cut pSP72 (Promega). This construct contains additional restriction sites flanking the PVY insert which were used to assist subsequent manipulations. A map of this construct is shown in Figure 45.

Plasmid ClapBC.PVY

Plasmid Cla pBC.PVY was prepared by inserting a ClaI/SalI fragment from pSP72.PVY

Printed: 22-04-2003 TIPA1 99910592

into ClaI/Sal I cutpBC (Stratagene). This construct contains additional restriction sites flanking the PVY insert which were used to assist subsequent manipulations. A map of this construct is shown in Figure 46.

Plasmid pBC.PVYx2

Plasmid pBC.PVYx2 contains two direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was generated by cloning an AccI/ClaI PVY fragment from pSP72.PVY into AccI cut pBC.PVY and is shown in Figure 47.

Plasmid pSP72.PVYx2

Plasmid pSP72.PVYx2 contains two direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was generated by cloning an AccI/ClaI PVY fragment from pBc.PVY into AccI cut pSP72.PVY and is shown in Figure 48.

Plasmid pBC.PVYx3

Plasmid pBC.PVYx3 contains three direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was prepared by cloning an AccI/ClaI PVY fragment from pSP72.PVY into AccI cut pBC.PVYx2 and is shown in Figure 49.

Plasmid pBC.PVYx4

Plasmid pBC.PVYx4 contains four direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was prepared by cloning the direct repeat of PVY sequences from pSP72.PVYx2 as an AccI/ClaI fragment into AccI cut pBC.PVYx2 and is shown in Figure 50.

Plasmid pBC.PVY.LNYV

All attempts to create direct palindromes of PVY sequences failed, presumably such sequence arrangements are unstable in commonly used E. coli cloning hosts. Interrupted palindromes however proved stable.

20-04-2003

Printed: 22-04-2005 TIPA1 99910592

To create interrupted palindromes of PVY sequences a "stuffer" fragment of approximately 360 bp was inserted into Cla pBV.PVY downstream of the PVY sequences. The stuffer fragment was made as follows:

A clone obtained initially from a cDNA library prepared from lettuce necrotic yellows virus (LNYV) genomic RNA (Deitzgen *et al*, 1989), known to contain the 4b gene of the virus, was amplified by PCR using the primers:

LNYV 1:5′-ATGGGATCCGTTATGCCAAGAAGAAGGA-3′; and

LNYV 2:5′-TGTGGATCCCTAACGGACCCGATG-3′

The first 9 nucleotide of these primers encode a BamHI site, the remaining nucleotides are homologous to sequences of the LNYV 4b gene.

Following amplification, the fragment was cloned into the EcoRI site of pCR2.1 (Stratagene). This EcoRI fragment was cloned into the EcoRI site of Cla pBC.PVY to create the intermediate plasmid pBC.PVY.LNYV which is shown in Figure 51.

Plasmid pBC.PVY.LNYV.PVY

The plasmid pBC.PVY.LNYV.YVP contains an interrupted direct repeat of PVY sequences. to create this plasmid a HpaI/HincII fragment from pSP72 was cloned into SmaI-digested pBC.PVY.LNYV and a plasmid containing the sense orientation isolated, a map of this construct is shown in Figure 52.

Plasmid pBC.PVY.LNYV.YVP$_\Delta$

The plasmid pBV.PVY.LNYV.YVP$_\Delta$ contains a partial interrupted palindrome of PVY sequences. One arm of the palindrome contains all the PVY sequences from pBC.PVY, the other arm contains part of the sequences from PVY, corresponding to sequences between the EcoRV and HincII sites of pSP72.PVY. To create this plasmid

20-04-2005

Printed: 22-04-2005 JIPA1 99910592

an EcoRV/HincII fragment from pSP72.PVY was cloned into SmaI-digested pBC.PVY.LNYV and a plasmid containing the desired orientation isolated, a map of this construct is shown in Figure 53.

Plasmid pBC.PVY.LNYV.YVP

The plasmid pBC.PVY.LNYV.YVP contains an interrupted palindrome of PVY sequences. To create this plasmid a HpaI/HincII fragment from pSP72. was cloned into Sma-digested pBC.PVY.LNYV and a plasmid containing the antisense orientation isolated, a map of this construct is shown in Figure 54.

5. Control plasmids

Plasmids pART7.PVY & pART7.PVY

Plasmid pART7.PVY (Figure 55) was designed to express PVY sequences driven by the 35S promoter. This plasmid serves as a control construct in these experiments, against which all other constructs was compared. To generate this plasmid a ClaI/AccI fragment from ClapBC.PVY was cloned into ClaI-digested pART7 and a plasmid with expected to express a sense PVY sequence with respect to the PVY genome, was selected. Sequences consisting of the 35S promoter, PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.

Plasmids pART7.35S.PVY.SCBV.O & pART27.35S.PVY.SCBV.O

Plasmid pART7.35S.PVY.SCBV.0 (Figure 56) was designed to act as a control for co-expression of multiple constructs from a single plasmid in transgenic plants. The 35S promoter was designed to express PVY sense sequences, whilst the SCBV promoter was empty. To generate this plasmid, the PVY fragment from Cla pBC.PVY was cloned as a XhoI/EcoRI fragment into XhoI/EcoRI-digested pART7.35S.SCBV.cass to create p35S.PVY.SCBV>O. Sequences consisting of the 35S promoter driving sense PVY sequences and the NOS terminator and the SCBV promoter and OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid with

20-04-2005

Printed: 22-04-2005 TIPA 99910592

the desired insert orientation was isolated and designated pART27.35S.PVY.SCBV.O.

Plasmids pART7.35S.O.SCBV.PVY & pART27.35S.O.SCBV.PVY

Plasmid pART27.35S.O.SCBV.PVY (Figure 57) was designed to act as an additional control for co-expression of multiple constructs from a single plasmid in transgenic plants. No expressible sequences were cloned behind the 35S promoter, whilst the SCBV promoter drove expression of a PVY sense fragment. To generate this plasmid, the PVY fragment from Cla pBC.PVY was cloned as a ClaI fragment into ClaI-digested pART7.35S.SCBV.cass, a plasmid containing PVY sequences in a sense orientation was isolated and designated p35S.O.SCBV.PVY. Sequences, consisting of the 35S promoter and NOS terminator, the SCBV promoter driving sense PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was isolated and designated pART27.35S.O.SCBV.PVY.

Plasmids pART7.35S.O.SCBV.YVP & pART7.35S.O.SCBV.YVP

Plasmid pART7.35S.O.SCBV.YVP (Figure 58) was designed to act as an additional control for co-expression of multiple constructs from a single plasmid in transgenic plants. No expressible sequences were cloned behind the 35S promoter, whilst the SCBV promoter drove expression of a PVY antisense fragment. To generate this plasmid, the PVY fragment from Cla pBC.PVY was cloned as a ClaI fragment into ClaI-digested p35S.SCBV.cass, a plasmid containing PCY sequences in an antisense orientation was isolated and designated p35S.O.SCBV.YVP. Sequences, consisting of the 35S promoter and NOS terminator, the SCBV promoter driving sense PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was isolated and designated pART27.35S.O.SCBV.YVP.

6. Test plasmids

Plasmids pART7.PVYx2 & pART27.PVYx2

20-04-2005

Printed: 22-04-2005 IPAU 99910592

Plasmid pART7.PVYx2 (Figure 59) was designed to express a direct repeat of PVY sequences driven by the 35S promoter in transgenic plants. To generate this plasmid, direct repeats from pBC.PVYx2 were cloned as a XhoI/BamHI fragment into XhoI/BamHI cut pART7. Sequences consisting of the 35 S promoter, direct repeats of PVY and the OCS terminator were excised as a NotI fragment from pART7.PVYx2 and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVYx2.

Plasmids pART7.PVYx3 & pART27.PVYx3

Plasmid pART7.PVYx3 (Figure 60) was designed to express a direct repeat of three PVY sequences driven by the 35S promoter in transgenic plants. To generate this plasmid, direct repeats from pBC.PVYx3 were cloned as a XhoI/BamHI fragment into XhoI/BamHI cut pART7. Sequences consisting of the 35S promoter, direct repeats of PVY and OCS terminator were excised as a NotI fragment from pART.PVYx3 and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVYx3.

Plasmids pART7.PVYx4 & pART27.PVYx4

Plasmid pART7.PVYx4 (Figure 61) was designed to express a direct repeat of four PVY sequences driven by the 35S promoter in transgenic plants. To generate this plasmid, direct repeats from pBC.PVYx4 were cloned as a XhoI/BamHI fragment into xhoI/BamHI cut pART7. Sequences consisting of the 35S promoter, direct repeats of PVY and the OCS terminator were excised as a NotI fragment from pART7.PVYx3 and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVYx3.

Plasmids pART7.PVY.LNYV.PVY & pART27.PVY.LNYV.PVY

Plasmid pART7.PVY.LNYV.PVY (Figure 62) was designed to express the interrupted direct repeat of PVY sequences driven by the 35S promoter in transgenic plants. This construct was prepared by cloning the interrupted direct repeat of PVY from

20-04-2005

Printed: 22-04-2005 IPA 99910592

pBC.PVY.LNYV.PVY as a XhoI/XbaI fragment into pART7 digested with XhoI/XbaI. Sequences consisting of the 35S promoter, the interrupted direct repeat of PVY sequences and the OCS terminator were excised from pART7.PVY.LNYV.PVY as a NotI fragment and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVY.LNYV.PVY.

Plasmids pART7.PVY.LNYV.YVP$_\Delta$ & pART27.PVY.LNYV.YVP$_\Delta$

Plasmid pART7.PVY.LNYV.YVP$_\Delta$ (Figure 63) was designed to express the partial interrupted palindrome of PVY sequences driven by the 35S promoter in transgenic plants. This construct was prepared by cloning the partial interrupted palindrome of PVY sequences from pBC.PVY.LNYV.YVP$_\Delta$ as a XhoI/XbaI fragment into pART7 digested with XhoI/XbaI. Sequences consisting of the 35S promoter, the partial interrupted palindrome of PVY sequences and the OCS terminator were excised from pART7.PVY.LNYV.YVP$_\Delta$ as a NotI fragment and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVY.LNYV.YVP.

Plasmids pART7.PVY.LNYV.YVP & pART27.PVY.LNYV.YVP

Plasmid pART7.PVY.LNYV.YVP (Figure 64) was designed to express the interrupted palindrome of PVY sequences driven by the 35S promoter in transgenic plants. This construct was prepared by cloning the interrupted palindrome of PVY sequences from pBC.PVY.LNYV.YVP$_\Delta$ as a XhoI/XbaI fragment into pART7 digested with XhoI/XbaI. Sequences consisting of the 35S promoter, the interrupted palindrome of PVY sequences and the OCS terminator were excised from pART7.PVY.LNYV.YVP as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVY.LNYV.YVP.

Plasmids pART7.35S.PVY.SCBV.YVP & pART27.35S.PVY.SCBV.YVP

Plasmid pART7.35S.PVY.SCBV.YVP (Figure 65) was designed to co-express sense and antisense constructs in transgenic plants. To generate this plasmid the PVY

212 20-04-2005

Printed: 22-04-2005 FIRST 99910592

fragment from Cla pBC.PVY was cloned as a XhoI/EcoRI fragment into xhoI/EcoRI-digested p35S.SCBV.O.SCBV.YVP. Sequences, consisting of the 35S promoter driving sense PVY sequences and the NOS terminator and the SCBV promoter driving antisense PVY and the OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was isolated and designated pART27.35S.PVY.SCBV.YVP.

Plasmids pART7.35S.PVYx3.SCBV.YVPx3 & pART27.35S.PVYx3.SCBV.YVPx3

Plasmid pART7.35S.PVYx3.SCBV.YVPx3 (Figure 66) was designed to co-express sense and antisense repeats of PVY in transgenic plants. to generate this plasmid, the intermediate pART7.35S.O.SCBV.YVPx3 was constructed by cloning the triple direct PVY repeat from ClapBC.PVYx3 as a ClaI/AccI fragment into Cla-digested p35S.SCBV.cass and isolating a plasmid with an antisense orientation. for p35S.PVYx3.SCBV.YVPx3 the triple direct PVY repeat from Cla pBC.PVYx3 was cloned as a KpnI/SmaI fragment into KpnI/SmaI-digested p35S.O.SCBV.YVPx3 to create p35S.PVYx3.SCBV.YVPx3. Sequences including both promoters, terminators and direct PVY repeats were isolated as a NotI fragment and cloned into pART27. A plasmid with an appropriate orientation was chosen and designated pART27.35S.PVYx3.SCBV.

Plasmids pART7.PVYx3.LNYV.YVPx3 & pART27.PVYx3.LNYV.YVPx3

Plasmid pART7.PVYx3.LNYV.YVPx3 (Figure 67) was designed to express triple repeats of PVY sequences as an interrupted palindrome. To generate this plasmid an intermediate, pART7x3.PVY.LNYV.YV was constructed by cloning a PVY.LNYV.YVP fragment from pBC.PVY.LNYV.YVP as an AccI/ClaI fragment into the plasmid pART7.PVYx2. pART7.35S.PVYx3.LNYV.YVPx3, was made by cloning an additional PVY direct repeat from pBC.PVYx2 as an AccI/ClaI fragment into ClaI digested pART7x3.PVY.LNYV.YVP. Sequences from pART7.35S.PVYx3.LNYV.YVPx3, including the 35S promoter, all PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into NotI-digested pART27, a plasmid with an

20-04-2005

appropriate orientation was chosen and designated pART27.35S.PVYx3.LNYV.

Plasmids pART7.PVY multi & pART27.PVY multi

Plasmid pART7.35S.PVY multi (Figure 68) was designed to express higher order direct repeats of regions of PVY sequences in transgenic plants. Higher order direct repeats of a 72 bp of the PVY Nia region from PVY were prepared by annealing two partially complementary oligonucleotides as follows:

PVY1:
5′-TAATGAGGATGATGTCCCTACCTTTAATTGGCAGAAATTTCTGTGGAAAGACAG GGAAATCTTTCGGCATTT-3′; and

PVY2:
5′-TTCTGCCAATTAAAGGTAGGGACATCATCCTCATTAAAATGCCGAAAGATT TCCCTGTCTTTCCACAGAAAT-3′

The oligonucleotides were phosphorylated with T4 polynucleotide kinase, heated and cooled slowly to permit self-annealing, ligated with T4 DNA ligase, end-filled with Klenow polymerase and cloned into pCR2.1 (Invitrogen). Plasmids containing multiple repeats were isolated and sequences were cloned as EcoRI fragments in a sense orientation into EcoRI-digested pART7, to create the intermediate pART7.PVY multi. to create pART27.PVY multi, the 35S promoter, PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into NotI-digested pART27. A plasmid with an appropriate insert orientation was isolated and designated pART27.PVY multi.

Printed: 22-04-2005 EPA 99910592

EXAMPLE 6

Inactivation of virus gene expression in mammals

Viral immune lines are created by expressing viral sequences in stably transformed cell lines.

In particular, lytic viruses are used for this approach since cell lysis provides very simple screens and also offer the ability to directly select for potentially rare transformation events which might create viral immunity. Sub-genomic fragments derived from a simple single stranded RNA virus (Bovine enterovirus - BEV) or a complex double stranded DNA virus, Herpes Simplex Virus I (HSV I) are cloned into a suitable vector and expressed in transformed cells. Mammalian cell lines are transformed with genetic constructs designed to express viral sequences driven by the strong cytomegalovirus (CMV-IE) promoter. Sequences utilised include specific viral replicase genes. Random "shotgun" libraries comprising representative viral gene sequences, may also be used and the introduced dispersed nucleic acid molecule, to target the expression of virus sequences.

Exemplary genetic constructs for use in this procedure, comprising nucleotide sequences derived from the BEV RNA-dependent RNA polymerase gene, are presented herein.

For viral polymerase constructs, large numbers (approximately 100) of transformed cell lines are generated and infected with the respective virus. For cells transformed with shotgun libraries very large numbers (hundreds) of transformed lines are generated and screened in bulk for viral immunity. Following virus challenge, resistant cell lines are selected and analysed further to determine the sequences conferring immunity thereon.

Resistant cell lines are supportive of the ability of the introduced nucleotide sequences to inactivate viral gene expression in a mammalian system.

20-04-2005

Additionally, resistant lines obtained from such experiments are used to more precisely define molecular and biochemical characteristics of the modulation which is observed.

EXAMPLE 8

Induction of virus resistance in transgenic plants

Agrobacterium tumefaciens, strain LBA4404, was transformed independently with the constructs pART27.PVY, pART27.PVYx2, pART27.PVYx3, pART27.PVYx4, pART27.PVY.LNYV.PVY, pART27.PVY.LNYV.YVP$_{\Delta}$, pART27.PVY.LNYV.YVP, pART27.35S.PVY.SCBV.O, pART27.35S.O.SCBV.PVY, pART27.35S.O.SCBV.YVP, pART27.35S.PVY.SCBV.YVP, pART27.35S.PVYx3.SCBV.YPVx3, pART27.PVYx3.LNYV.YVPx3 and pART27.PVYx10, using tri-parental matings. DNA mini-preps from these strains were prepared and examined by restriction with NotI to ensure they contained the appropriate binary vectors.

Nicotiana tabaccum (cultivar W38) were transformed with these *Agrobacterium* strains using standard procedures. Putative transformed shoots were excised and rooted on media containing kanamycin. Under these conditions we have consistently observed that only transgenic shoots will root on kanamycin plates. Rooted shoots were transferred to soil and allowed to establish. After two to three weeks, vigorous plants with at least three sets of leaves were chosen and infected with PVY.

Viral inoculum was prepared from W38 tobacco previously infected with the virus, approximately 2 g of leaf material, showing obvious viral symptoms were ground with carbarundum in 10 ml of 100mM Na phosphate buffer (pH 7.5). the inoculum was diluted to 200 ml with additional Na phosphate buffer. Two leaves from each transgenic plant were sprinkled with carbarundum, then 0.4 ml of inoculum was applied to each leaf and leaves rubbed fairly vigorously with fingers. Using this procedure 100% of non-transgenic control plants were infected with PVY.

Printed: 22-04-2005 TIPAT 99910592

To assay for viral resistance and immunity transgenic plants are monitored for symptom development. The PVY strain (PVY-D, an Australian PVY isolate) gives obvious symptoms on W38 tobacco, a vein clearing symptom is readily observed on the two leaves above the inoculated leaves, subsequent leaves show uniform chlorotic lesions. Symptom development was monitored over a six week period.

Transgenic lines were described as resistant if they showed reduced viral symptoms, which manifests as a reduction in the leaf are showing chlorotic lesions. Resistance ranges from very strong resistance where only a few viral lesions are observed on a plant to weak resistance which manifects as reduced symptoms on leaves that develop late in plant growth.

Transgenic plants which showed absolutely no evidence of viral symptoms were classified as immune. To ensure these plants were immune they were re-inoculated with virus, most plants remained immune, the few that showed symptoms were re-classified as resistant.

For plant lines generated Southern blots are performed, resistance in subsequent generations is monitored to determine that resistance/immunity is transmissable. Additionally, the breadth of viral resistance is monitored by challenging lines with other PVY strains, to determine whether host range susceptibility is modified.

Results from these experiments are described in Table 2 . These data indicate that constructs comprising tandem repeats of target gene sequence, either in the configuration of palindromes, interrupted palindromes as direct repeat sequences, are capable of conferring viral resistance and/or immunity in transgenic plants.

Accordingly, such inverted and/or direct repeat sequences modulate expression of the virus target gene in the transgenic plant.

20-04-2005

Constructs combining the use of direct and inverted repeat sequences, namely pART27.35S.PVYx3.SCBV.YVPx3 and pART27.PVYx3.LNYV.YVPx3, are also useful in modulating gene expression.

EXAMPLE 9

Inactivation of Galt in animal cells

To assay for Galt inactivation, porcine PK2 cells were transformed with the relevant constructs. PK2 cells constitutively express Galt enzyme, the activity of which results in the addition of a variety of α-1,3-galactosyl groups to a range of proteins expressed on the cell surface of these cells. Cells were transformed using lipofectin and stably transformed lines were selected using genetecin.

As an initial assay cell lines were probed for the presence of the Galt-encoded epitope, i.e. α-1,3-galactosyl moieties decorating cell surface proteins, using the lectin IB4. IB4 binding was assayed either *in situ* or by FACS sorting.

For *in situ* binding, cells were fixed to solid supports with cold methanol for 5 mins, cells were rinsed in PBS (phosphate buffered saline) and non-specific IB4 binding was blocked with 1% BSA in PBS for 10 mins. Fixed cells were probed using 20 ug/ml IB4-biotin (Sigma) in 1% BSA, PBS for 30 mins at room temperature, cells were washed in PBS then probed with a 1:200 dilution of ExtrAvidin-FITC (Sigma) in PBS for 30 mins followed by further rinses in PBS. Cells were then examined using fluorescence microscopy, under these conditions the outer surface of PK2 control cells uniformly stained green.

For FACS analysis, cells were suspended after treatment with trypsin, washed in HBSS/Hepes (Hank's buffered saline solution with 20 mM Hepes, pH7.4) and probed with 10 ug/ml IB4-biotin (Sigma) in HBSS/Hepes for 45 mins at 4°C. Cells were washed in HBSS/Hepes, probed with a 1:200 dilution of ExtrAvidin-FITC (Sigma) in HBSS/Hepes for 45 mins at 4°C at and rinsed in cold HBSS/Hepes prior to FACS

Printed 22-04-2005 TIPA 99910592

sorting.

Using this approach transformed cell lines are assayed for Galt inactivation and quantitative assessment of construct effectiveness is determined. Moreover cell lines showing Galt inactivation are isolated and subject to further molecular analyses to determine the mechanism of gene inactivation.

20-04-2005

220
Printed: 22-04-2005
WO 99/49029
TIPAT
- 72 -
PCT/AU99/00195
99910592
20-04-2005

PLASMID CONSTRUCT	No. OF PLANTS TESTED	PERCENTAGE OF PLANTS SHOWING SPECIFIED PHENOTYPE		
		SUSCEPTIBLE	IMMUNE	RESISTANT
pART27.PVY	19	16	1	2
pART27.PVYx2	13	5	4	4
pART27.PVYx3	21	2	5	14
pART27.PVYx4	21	5	7	9
pART27.35S.PVY.SCBC.0	25	8	0	17
pART27.35S.O.SCBV.PVY	22	8	0	14
pART27.35S.O.SCBV.YVP	18	14	0	4
pART27.35S.PVY.SCBV.YVP	17	3	8	6
pART27.PVY.LNYV.PVY	26	18	2	6
pART27.PVY.LNYV.YVP	20	6	10	4
pART27.PVY.LNYV.YVP$_{\Delta}$	18	7	11	0

Printed: 22-04-2005
IPA1
99910592

REFERENCES

1. An *et al.* (1985) *EMBO J 4*:277-284.
2. Armstrong, *et al.Plant Cell Reports 9:* 335-339, 1990.
3. Ausubel, F.M. *et al.*(1987) *In:* Current Protocols in Molecular Biology, Wiley Interscience (ISBN 047140338)..
4. Chalfie,M. *et al* (1994) *Science* **263**: 802-805.
5. Christensen, A.H. and Quail, P.H. (1996) *Transgenic Research* **5**: 213-218.
6. Christou, P., *et al. Plant Physiol 87:* 671-674, 1988.
7. Cormack, B. *et al* (1996) *Gene* **173**: 33-38.
8. Crossway *et al., Mol. Gen. Genet. 202:*179-185, 1986.
9. Dorer, D.R., and Henikoff, S. (1994) *Cell* **7**: 993-1002.
10. Fromm *et al. Proc. Natl. Acad. Sci. (USA) 82:*5824-5828, 1985.
11. Gleave, A.P. (1992) *Plant Molecular Biology* **20**:1203-1207.
12. Hanahan, D. (1983) *J. Mol.Biol.* **166**: 557-560.
13. Herrera-Estella *et al.*, *Nature 303:* 209-213, 1983a.
14. Herrera-Estella *et al.*,*EMBO J. 2:* 987-995, 1983b.
15. Herrera-Estella *et al. In:* Plant Genetic Engineering, Cambridge University Press, N.Y., pp 63-93, 1985.
16. Inouye, S. and Tsuji, F.I. (1994) *FEBS Letts.* **341**: 277-280.
17. Jackson, I.J. (1995) *Ann. Rev. Genet.* **28**: 189-217.
18. Krens, F.A., *et al., Nature 296:* 72-74, 1982.
19. Kwon, B.S. *et al.* (1988) *Biochem. Biophys. Res. Comm.* **153**:1301- 1309.
20. Pal-Bhadra, M. *et al.* (1997) *Cell* **90**: 479-490.
21. Paszkowski *et al.*, *EMBO J. 3*:2717-2722, 1984
22. Prasher, D.C. *et al.* (1992) *Gene* **111**: 229-233.
23. Sanford, J.C., *et al., Particulate Science and Technology 5:* 27-37, 1987.

221
20-04-2005

Printed: 22-04-2005 TIPA1 99910592

CLAIMS

1. A method of repressing, delaying or otherwise reducing the expression of a target gene in a cell, tissue or organ, said method comprising introducing to said cell, tissue or organ one or more dispersed nucleic acid molecules or foreign nucleic acid molecules comprising multiple copies of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or complementary thereto for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

2. The method according to claim 1 wherein the dispersed nucleic acid molecules or foreign nucleic acid molecules comprise inverted repeats of the target gene sequence or a region thereof or complementary thereto.

3. The method according to claim 1 wherein the dispersed nucleic acid molecules or foreign nucleic acid molecules comprise direct repeats of the target gene sequence or a region thereof or complementary thereto.

4. The method according to claim 1 wherein the dispersed nucleic acid molecules or foreign nucleic acid molecules comprise both direct and inverted repeats of the target gene sequence or a region thereof or complementary thereto.

5. The method according to any one of claims 1 to 4, wherein the number of copies of the target gene sequence or region thereof or complementary thereto in the dispersed nucleic acid molecule or foreign nucleic acid molecule is two.

6. The method according to any one of claims 1 to 4, wherein the number of copies of the target gene sequence or region thereof or complementary thereto in the dispersed nucleic acid molecule or foreign nucleic acid molecule is three.

222 20-04-2005

7. The method according to any one of claims 1 to 4, wherein the number of copies of the target gene sequence or region thereof or complementary thereto in the dispersed nucleic acid molecule or foreign nucleic acid molecule is four.

8. The method according to any one of claims 1 to 4, wherein the number of copies of the target gene sequence or region thereof or complementary thereto in the dispersed nucleic acid molecule or foreign nucleic acid molecule is six.

9. The method according to any one of claims 1 to 4, wherein the number of copies of the target gene sequence or region thereof or complementary thereto in the dispersed nucleic acid molecule or foreign nucleic acid molecule is ten.

10. The method according to any one of claims one to 9 wherein the dispersed nucleic acid molecule or foreign nucleic acid molecule comprises tandem repeats of the target gene sequence and wherein one or more of the repeated units of said tandem repeats is separated from another unit be a nucleic acid-containing stuffer fragment.

11. The method according to any one of claims 1 to 10 wherein the cell, tissue or organ is an animal cell, tissue or organ.

12. The method according to claim 11 wherein the animal is a mouse.

13. The method according to any one of claims 1 to 10 wherein the cell, tissue or organ is a plant cell, tissue or organ.

14. The method according to claim 13 wherein the plant is a tobacco plant.

15. The method according to any one of claims 1 to 14 wherein the target gene is a gene which is contained within the genome of the cell, tissue or organ.

16. The method according to claim 15 wherein the target gene is α-1,3-galactosyltransferase.

17. The method according to any one of claims 1 to 14 wherein the target gene is derived from the genome of a pathogen of the cell, tissue or organ or an organism comprising said cell, tissue or organ.

18. The method according to claim 17 wherein the pathogen is a virus.

19. The method according to claim 18 wherein the virus is an animal pathogen.

20. The method according to claim 19 wherein the virus is BEV.

21. The method according to claim 18 wherein the virus is a plant pathogen.

22. The method according to claim 21 wherein the virus is PVY.

23. The method according to any one of claims 1 to 22 further comprising selecting the dispersed nucleic acid molecule(s) or foreign nucleic acid molecule(s) according to their ability to effectively modulate expression of the target gene.

24. A method of repressing, delaying or otherwise reducing the expression of a target gene in a cell, tissue or organ, said method comprising:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise tandem repeats of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or which is complementary thereto;

(ii) producing a synthetic gene comprising said dispersed nucleic acid molecules or foreign nucleic acid molecules operably connected to a promoter sequence operable in said cell, tissue or organ;

Printed: 22-04-2005 IIPA1 99910592

(iii) introducing said synthetic gene to said cell, tissue or organ; and

(iv) expressing said synthetic gene in said cell, tissue or organ for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

25. A method of conferring resistance or immunity to a viral pathogen upon a cell, tissue, organ or whole organism, comprising introducing one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise tandem repeats of a nucleotide sequence derived from the viral pathogen or a complementary sequence thereto for a time and under conditions sufficient for translation of the mRNA product of a virus gene to be delayed or otherwise reduced, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

26. The method according to claim 25 wherein the viral pathogen is a plant pathogen.

27. The method according to claim 26 wherein the virus is PVY.

28. The method according to claim 25 wherein the virus is an animal pathogen.

29. The method according to claim 28 wherein the virus is BEV.

30. The method according to any one of claims 25 to 29 further comprising selecting the dispersed nucleic acid molecule(s) or foreign nucleic acid molecule(s) according to their ability to confer resistance or immunity on the cell, tissue, organ or organism.

31. A method of conferring resistance or immunity to a viral pathogen upon a cell, tissue, organ or whole organism, comprising:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic

225 20-04-2005

acid molecules which comprise tandem repeats of a nucleotide sequence derived from the viral pathogen or a complementary sequence thereto;

(ii) producing a synthetic gene comprising said dispersed nucleic acid molecules or foreign nucleic acid molecules operably connected to a promoter sequence operable in said cell, tissue, organ or whole organism;

(iii) introducing said synthetic gene to said cell, tissue, organ or whole organism; and

(iv) expressing said synthetic gene in said cell, tissue or organ for a time and under conditions sufficient for translation of the mRNA product of a gene of the virus to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

32. The method according to any one of claims 25 to 31, wherein the dispersed nucleic acid molecules or foreign nucleic acid molecules comprise multiple copies of nucleotide sequence encoding a viral replicase, polymerase, coat protein or uncoating gene.

33. The method according to claim 32 wherein the dispersed nucleic acid molecules or foreign nucleic acid molecules comprise multiple copies of nucleotide sequence encoding a viral polymerase.

34. The method according to claim 32 wherein the dispersed nucleic acid molecules or foreign nucleic acid molecules comprise multiple copies of nucleotide sequence encoding a viral coat protein.

35. A synthetic gene which is capable of repressing, delaying or otherwise reducing the expression of a target gene in a cell, tissue, organ or whole organism, wherein said synthetic gene comprises a dispersed nucleic acid molecule or a foreign nucleic acid molecule comprising multiple copies of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a derivative thereof or a

complementary sequence thereto placed operably under the control of a promoter sequence which is operable in said cell, tissue, organ or whole organism.

36. The synthetic gene according to claim 35, wherein the dispersed nucleic acid molecule or a foreign nucleic acid molecule comprises tandem inverted and/or direct repeats of a genetic sequence that is endogenous to the genome of the cell, tissue, organ or organism or which is derived from a non-endogenous gene of the cell, tissue, organ or organism.

37. The synthetic gene according to claim 36 wherein the non-endogenous gene is derived from a viral pathogen of the cell, tissue, organ or organism.

38. The synthetic gene according to claim 37 wherein the non-endogenous gene is derived from an animal virus.

39. The synthetic gene according to claim 38 wherein the animal virus is BEV.

40. The synthetic gene according to claim 38 wherein the non-endogenous gene is derived from the BEV polymerase gene.

41. The synthetic gene according to claim 40 wherein the promoter is the CMV-IE promoter or SV40 promoter sequence.

42. The synthetic gene according to claim 37 wherein the non-endogenous gene is derived from a plant virus.

43. The synthetic gene according to claim 42 wherein the plant virus is PVY.

44. The synthetic gene according to claim 43 wherein the promoter is the CaMV 35S promoter or the SCBV promoter sequence.

Printed: 22-04-2005 TIPAT 99910592

45. The synthetic gene according to claims 35 or 36 wherein the dispersed nucleic acid molecule or a foreign nucleic acid molecule comprises tandem inverted and/or direct repeats of the porcine α-1,3-galactosyltransferase gene.

46. The synthetic gene according to claim 45 wherein the porcine α-1,3-galactosyltransferase gene is placed operably in connection with the CMV promoter sequence.

47. The synthetic gene according to any one of claims 35 to 46 wherein the multiple copies of the nucleotide sequence of the target gene are operably connected to two or more promoter sequences.

48. The synthetic gene according to claim 47 wherein each of the multiple copies of the nucleotide sequence of the target gene are operably connected to spatially separate promoter sequences.

49. A genetic construct comprising the synthetic gene according to any one of claims 35 to 48.

50. The genetic construct according to claim 49 selected from the list comprising plasmid pCMV.BEVx2; plasmid pCMV.BEV.GFP.VEB; plasmid pCMV.BEV.SV40L.BEV; and plasmid pCMV.BEV.SV40L.VEB.

51. The genetic construct according to claim 49 selected from plasmid pCMV.Galtx2; and pCMV.Galtx4.

52. The genetic construct according to claim 49 selected from the list comprising plasmid pSP72.PVYx2; plasmid pBC.PVYx2; plasmid pBC.PVYx3; plasmid pBC.PVYx4; plasmid pART27.PVYx2; plasmid pART27.PVYx3; plasmid pART27.PVYx4; plasmid pBC.PVY.LNYV.YVP$_{\Delta}$; plasmid pBC.PVY.LNYV.YVP;

228 20-04-2005

plasmid pBC.PVY.LNYV.PVY; plasmid pART27.PVY.LNYV.PVY; plasmid pART27.PVY.LNYV.YVP$_{\Delta}$; plasmid pART27.PVY.LNYV.YVP; plasmid pART27.35S.PVY.SCBV.YVP; plasmid pART27.35S.PVYx3.SCBV.YVPx3; plasmid pART27.PVYx3.LNYV.YVPx3; and plasmid pART27.PVYx10.

53. Use of the genetic construct according to claim 50 to confer immunity or resistance against BEV upon an animal cell, tissue or organ or a whole animal.

54. Use of the genetic construct according to claim 51 to delay, repress or otherwise reduce expression of α-1,3-galactosyltransferase in a cell, tissue, organ or whole organism that would otherwise express same.

55. Use of the genetic construct according to claim 52 to confer immunity or resistance against PVY upon a plant cell, tissue, organ or whole plant.

56. Use according to claim 55, wherein the plant is tobacco.

57. A cell, tissue, organ or whole organism comprising the synthetic gene according to any one of claims 35 to 48 or the genetic construct according to any one of claims 49 to 52 .

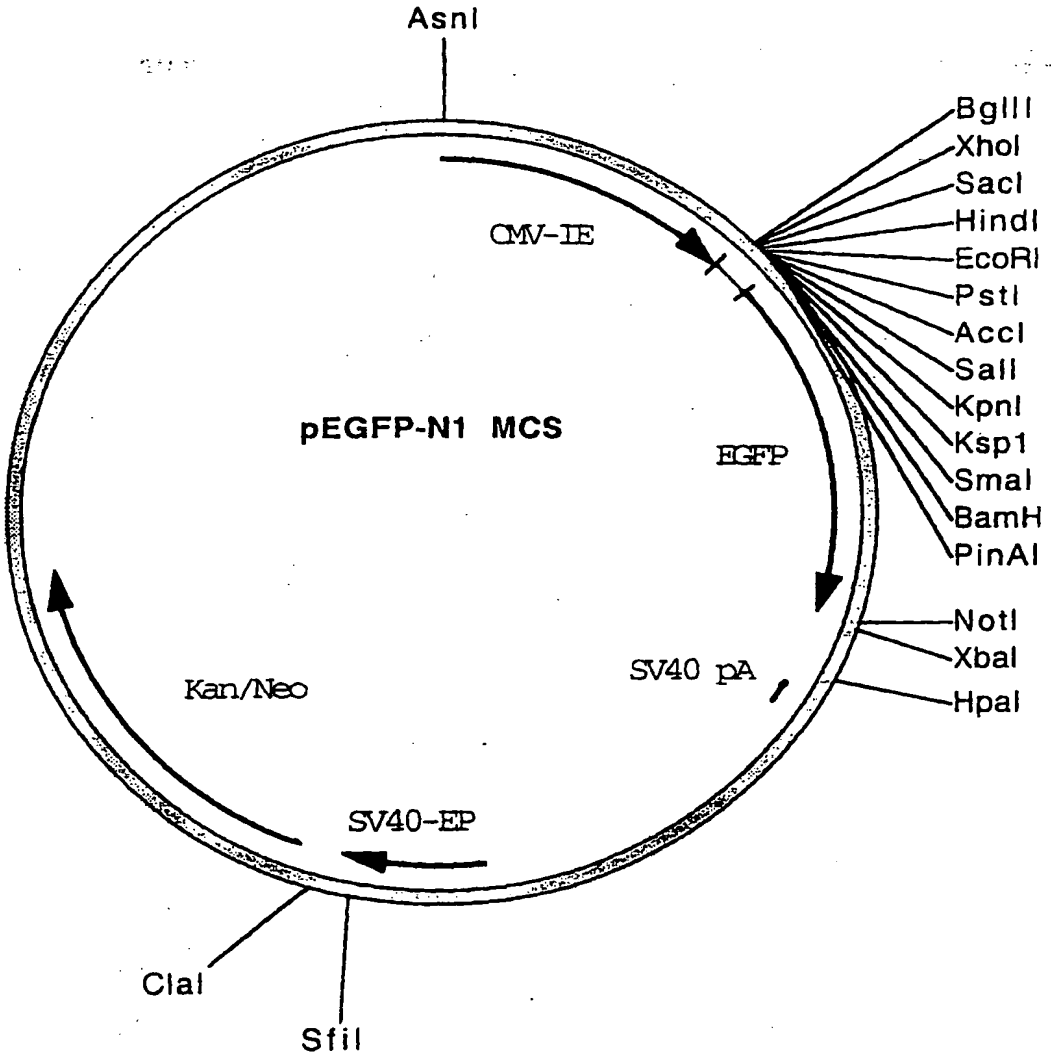

AsnI
BglII
XhoI
SacI
HindI
EcoRI
PstI
AccI
SalI
KpnI
Ksp1
SmaI
BamH
PinAI
NotI
XbaI
HpaI
CMV-IE
pEGFP-N1 MCS
EGFP
SV40 pA
Kan/Neo
SV40-EP
ClaI
SfiI

FIGURE 1

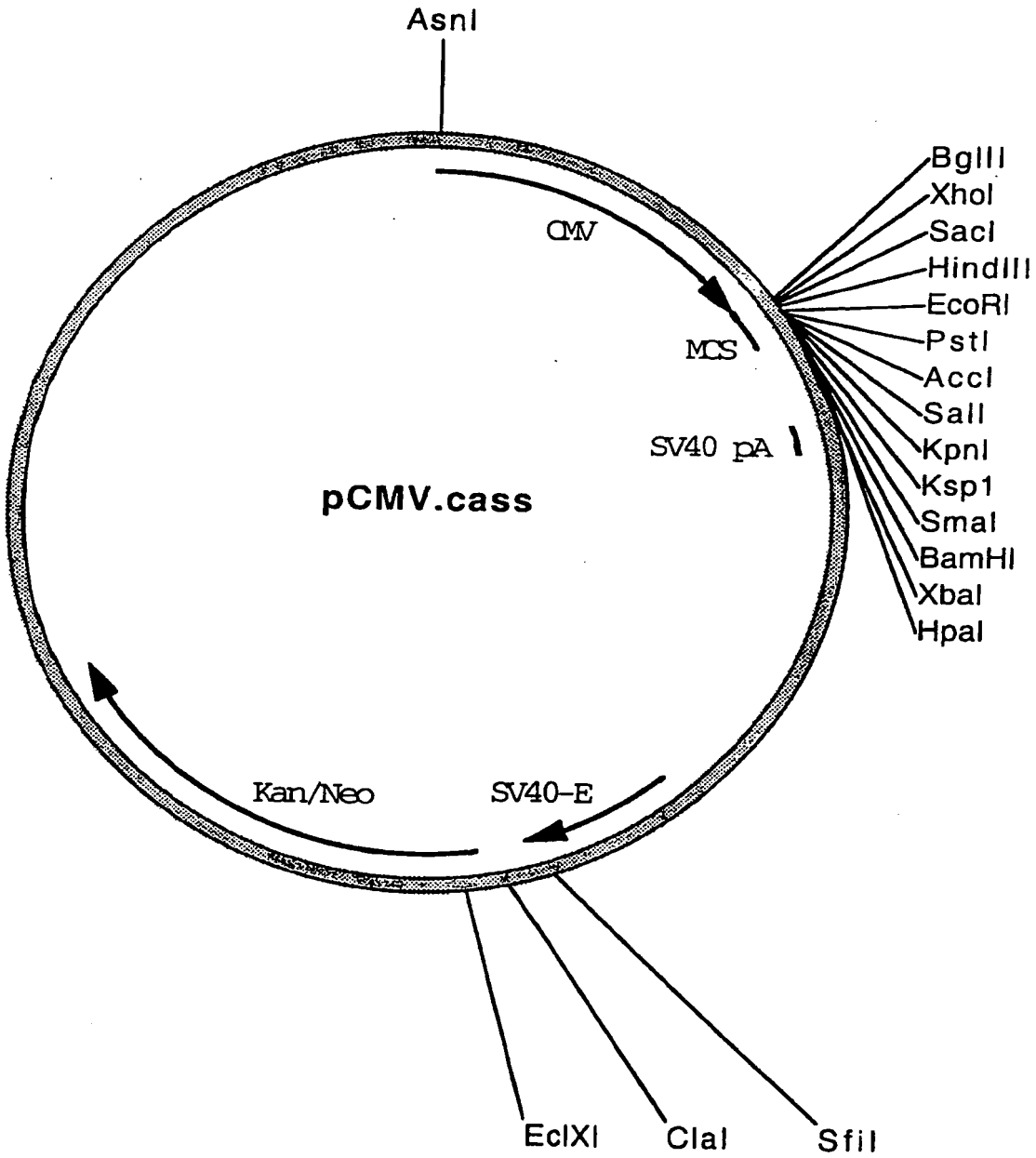

AsnI
BglII
XhoI
SacI
HindIII
EcoRI
PstI
AccI
SalI
KpnI
Ksp1
SmaI
BamHI
XbaI
HpaI
CMV
MCS
SV40 pA
pCMV.cass
Kan/Neo
SV40-E
EclXI
ClaI
SfiI

FIGURE 2

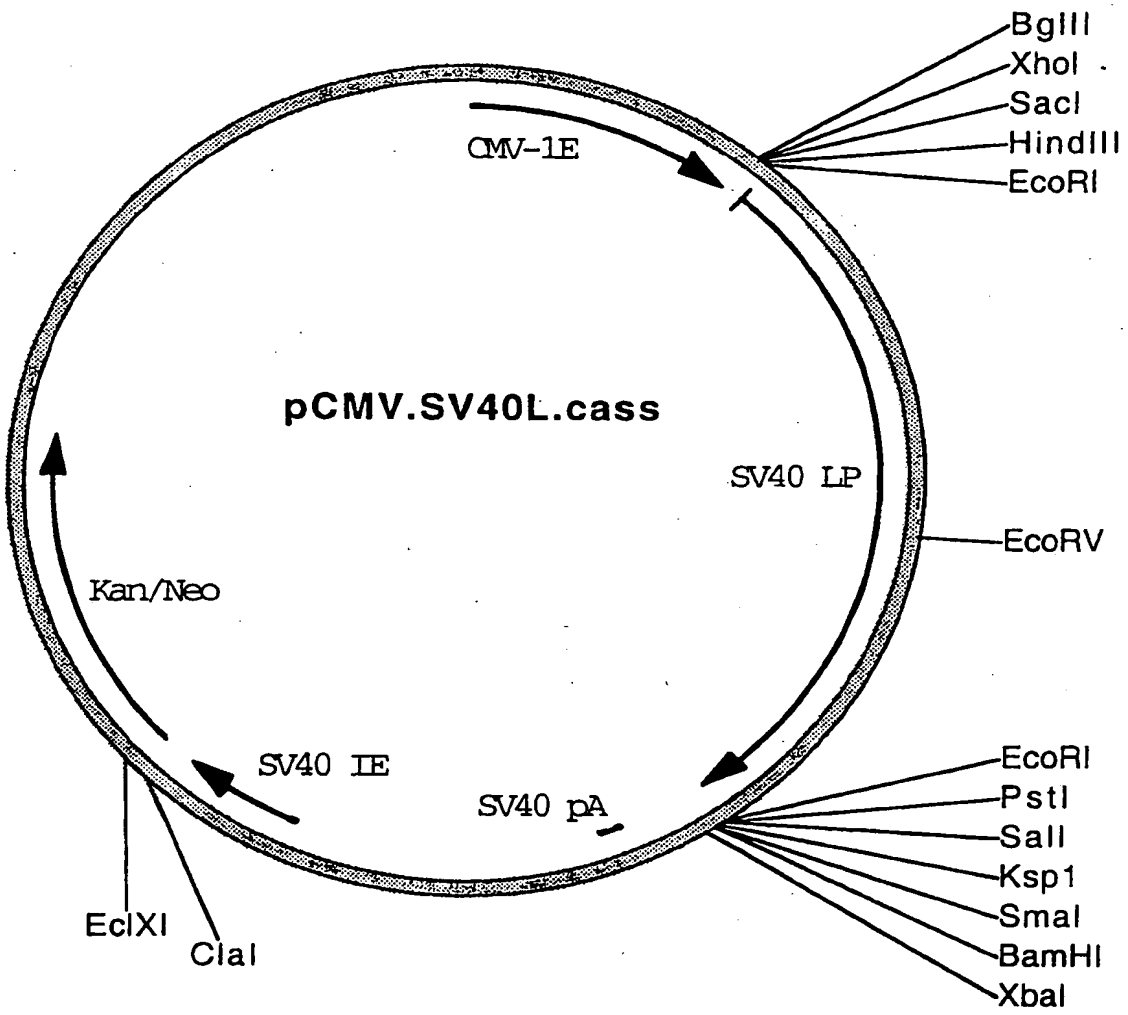

BglII
XhoI
SacI
HindIII
EcoRI
CMV-1E
pCMV.SV40L.cass
SV40 LP
EcoRV
Kan/Neo
SV40 IE
SV40 pA
EcoRI
PstI
SalI
Ksp1
SmaI
BamHI
XbaI
EclXI
ClaI

FIGURE 3

Printed: 22-04-2005 TIRA 99910592

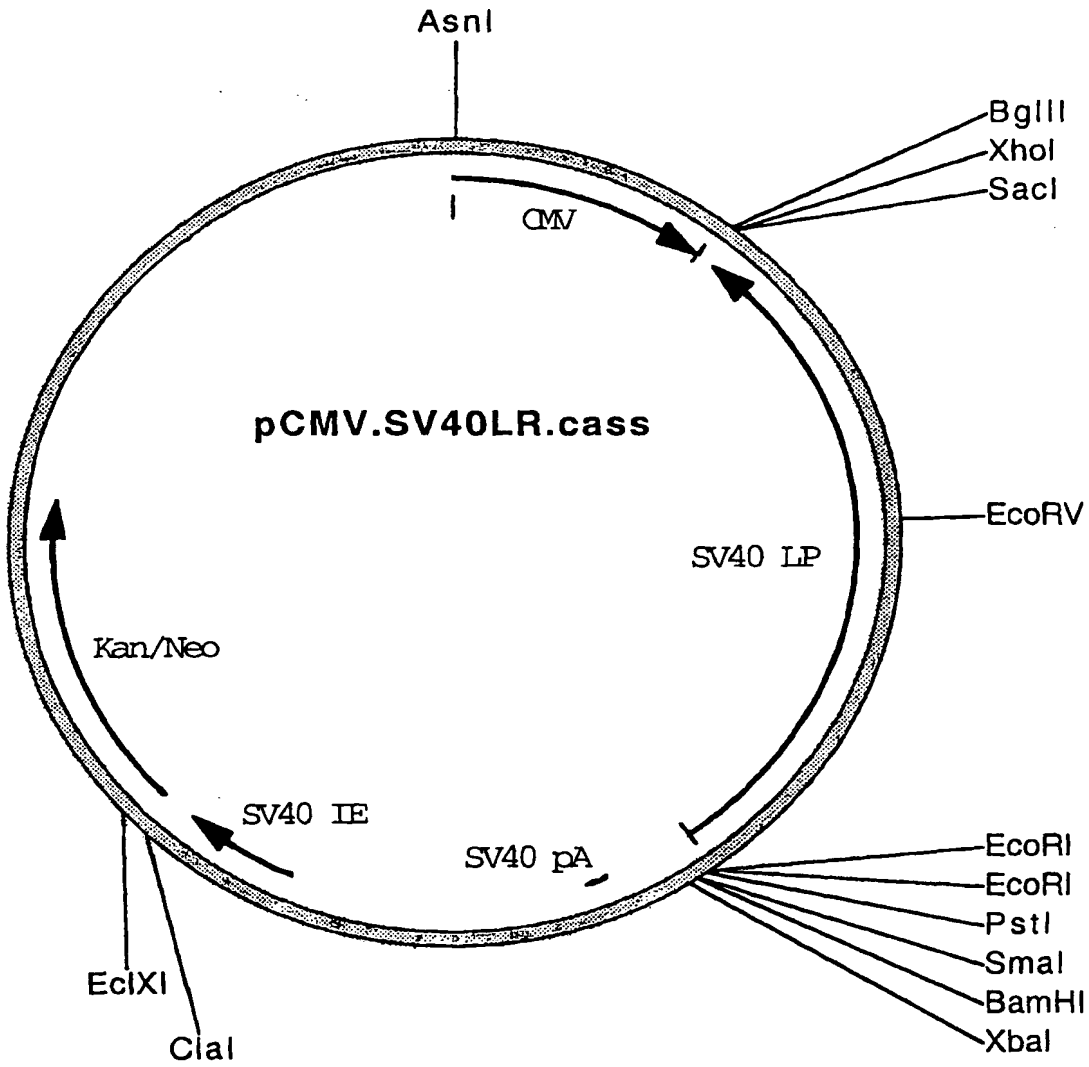


FIGURE 4

20-04-2005

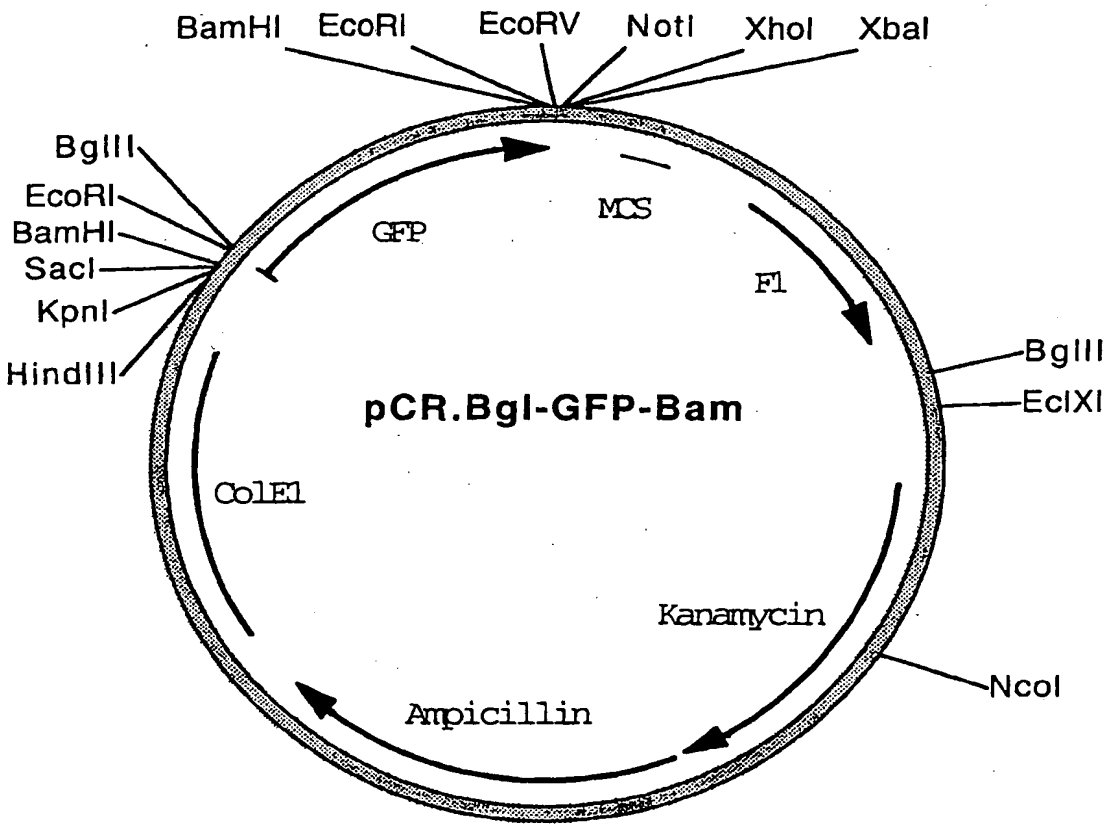

BamHI
EcoRI
EcoRV
NotI
XhoI
XbaI
BglII
EcoRI
BamHI
SacI
KpnI
HindIII
GFP
MCS
F1
BglII
EclXI
pCR.Bgl-GFP-Bam
ColE1
Kanamycin
NcoI
Ampicillin

FIGURE 5

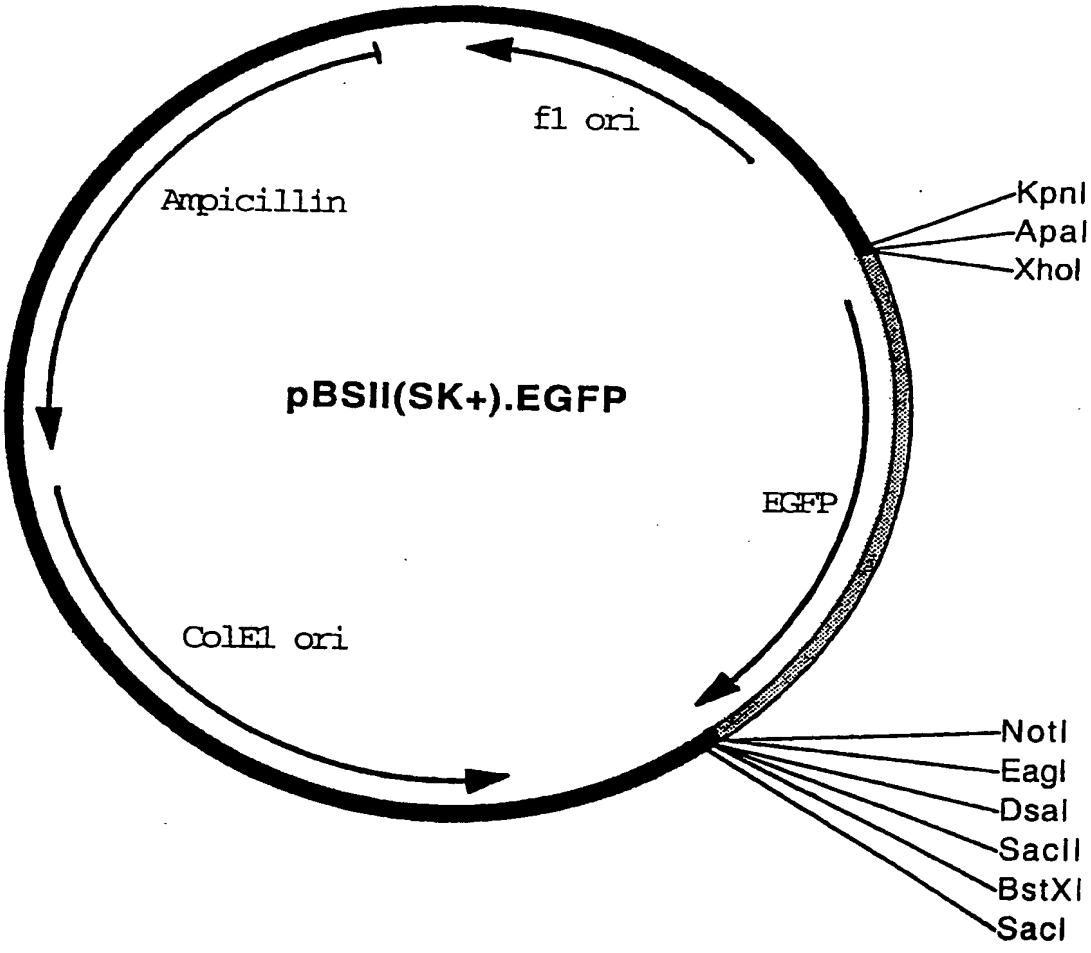

f1 ori
Ampicillin
KpnI
ApaI
XhoI
pBSII(SK+).EGFP
EGFP
ColE1 ori
NotI
EagI
DsaI
SacII
BstXI
SacI

FIGURE 6

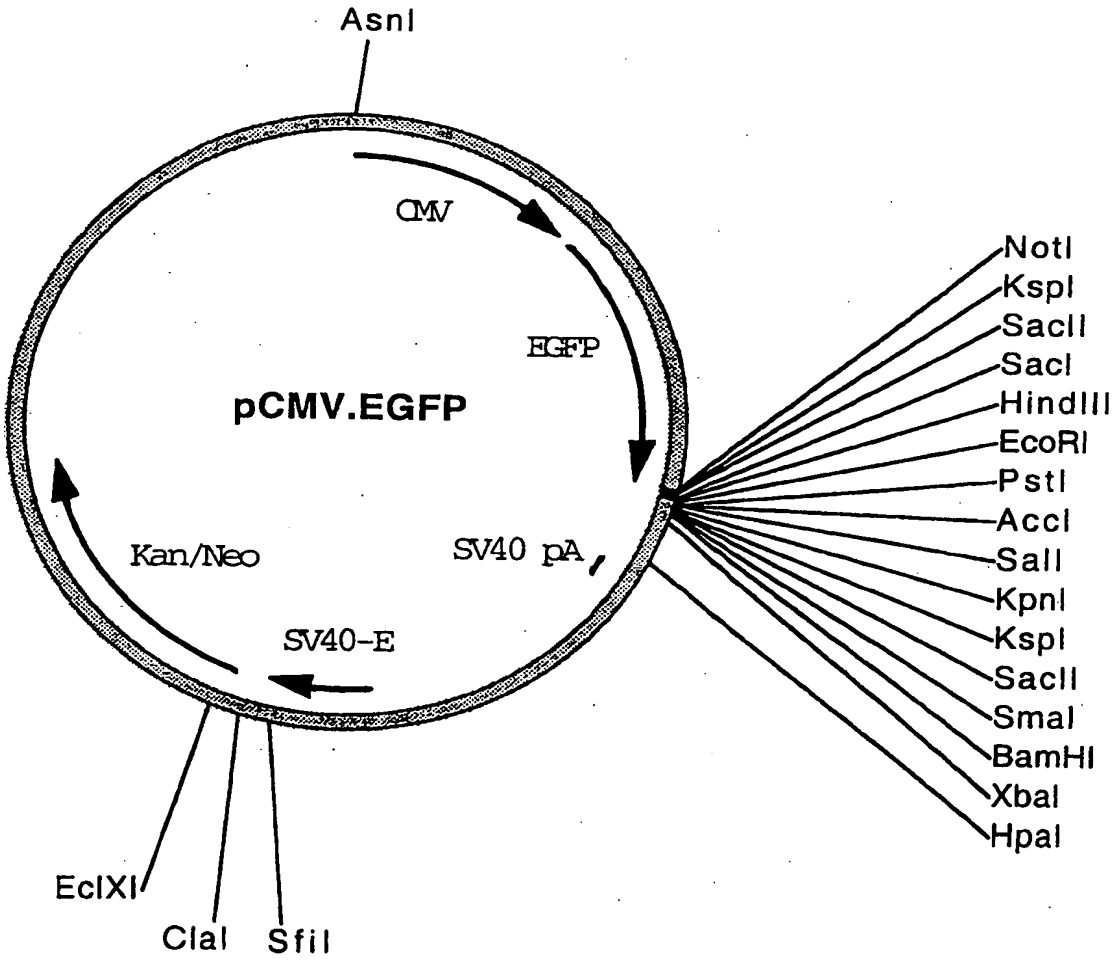

AsnI
CMV
EGFP
pCMV.EGFP
Kan/Neo
SV40 pA
SV40-E
NotI
KspI
SacII
SacI
HindIII
EcoRI
PstI
AccI
SalI
KpnI
KspI
SacII
SmaI
BamHI
XbaI
HpaI
EclXI
ClaI
SfiI

FIGURE 7

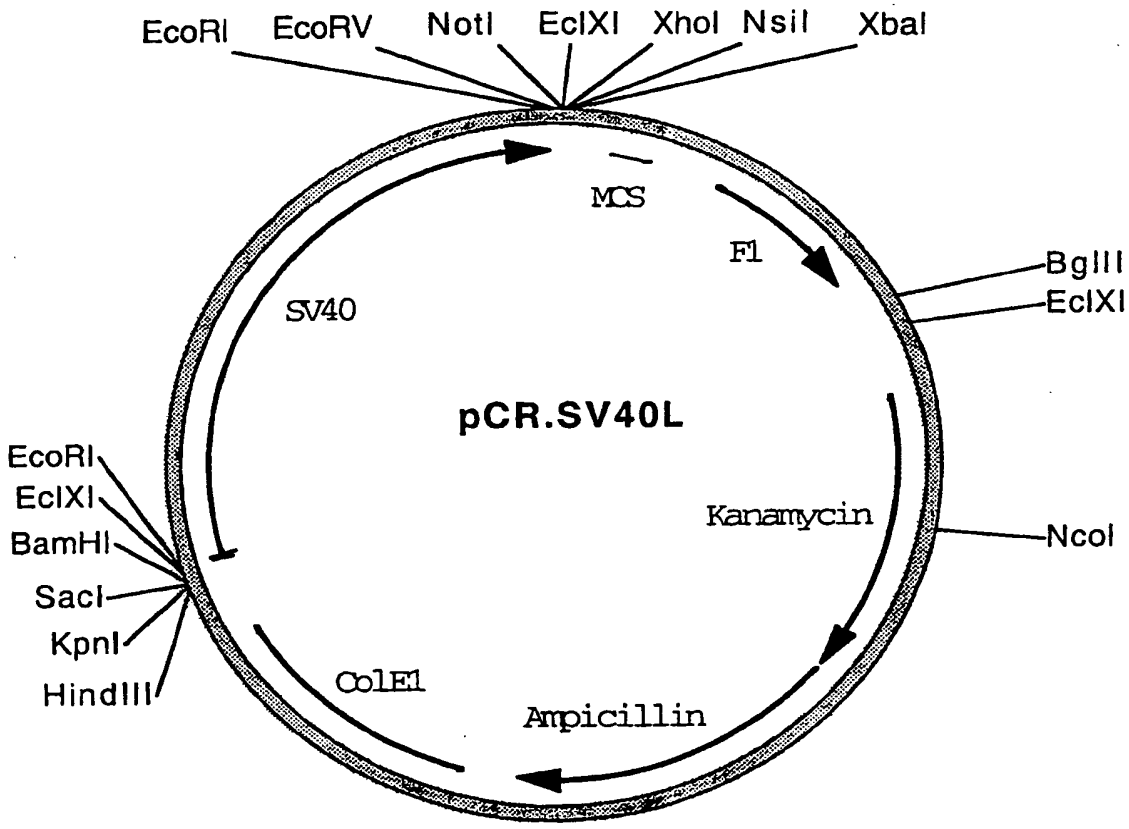

EcoRI
EcoRV
NotI
EclXI
XhoI
NsiI
XbaI
MCS
F1
BglII
EclXI
SV40
pCR.SV40L
EcoRI
EclXI
BamHI
SacI
KpnI
HindIII
Kanamycin
NcoI
ColE1
Ampicillin

FIGURE 8

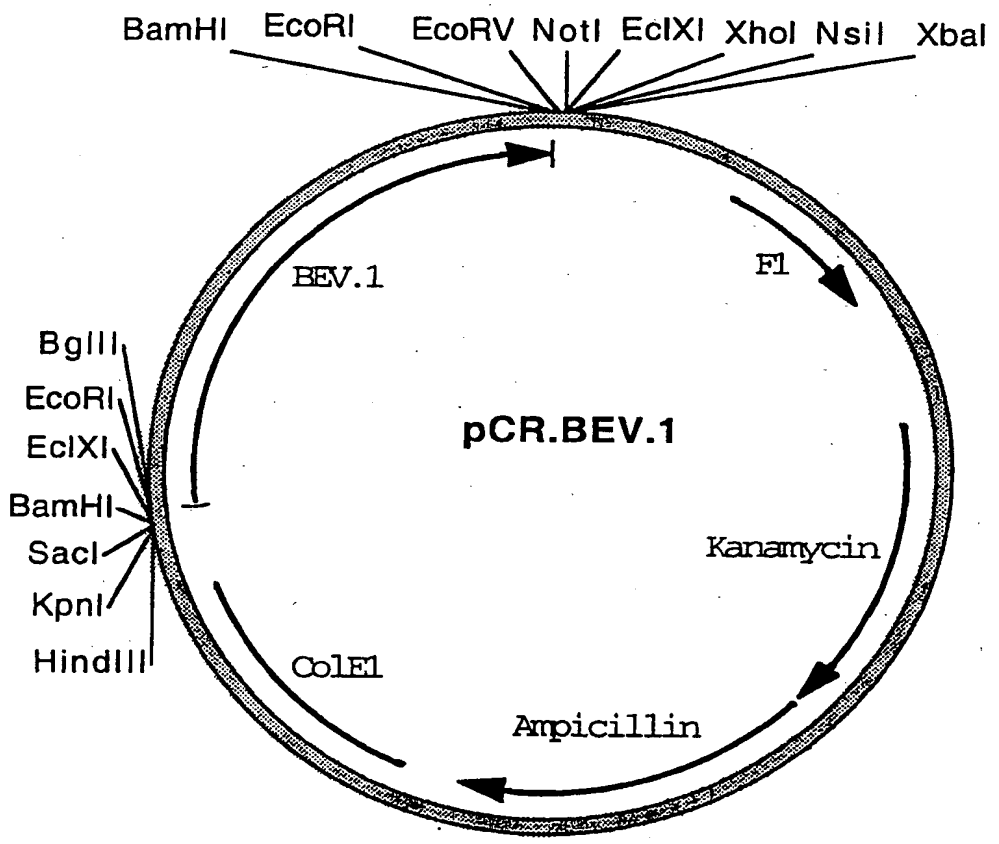

BamHI
EcoRI
EcoRV
NotI
EclXI
XhoI
NsiI
XbaI
BEV.1
F1
pCR.BEV.1
BglII
EcoRI
EclXI
BamHI
SacI
KpnI
HindIII
Kanamycin
ColE1
Ampicillin

FIGURE 9

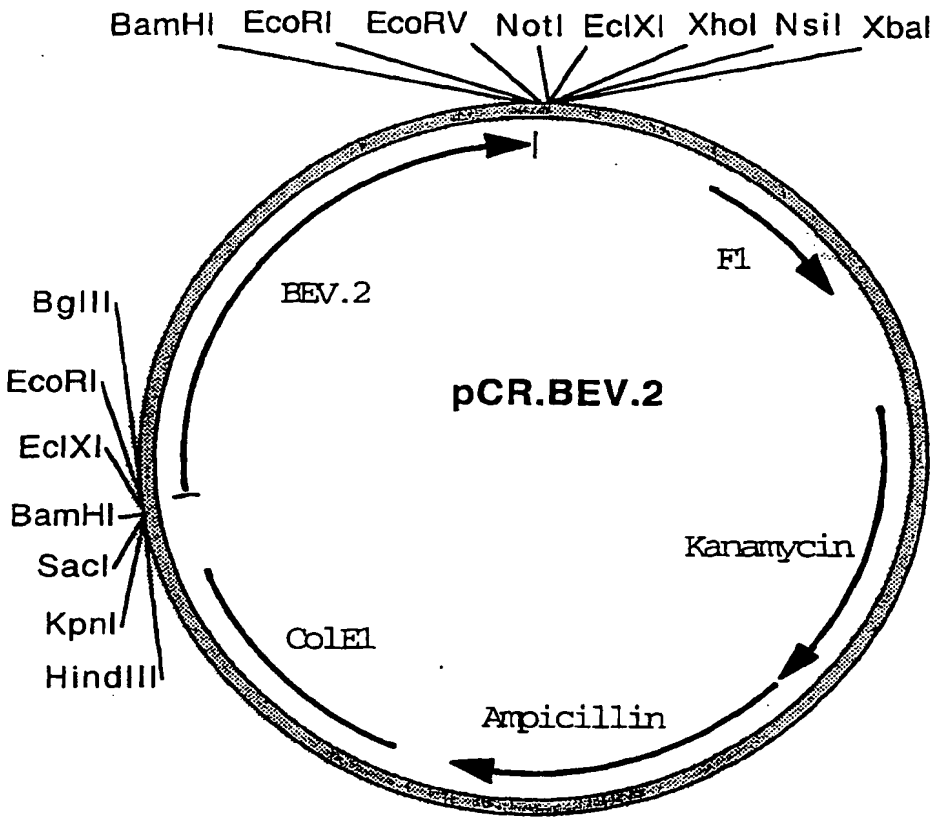

BamHI EcoRI EcoRV NotI EclXI XhoI NsiI XbaI
BglII
EcoRI
EclXI
BamHI
SacI
KpnI
HindIII
BEV.2
pCR.BEV.2
F1
Kanamycin
ColE1
Ampicillin

FIGURE 10

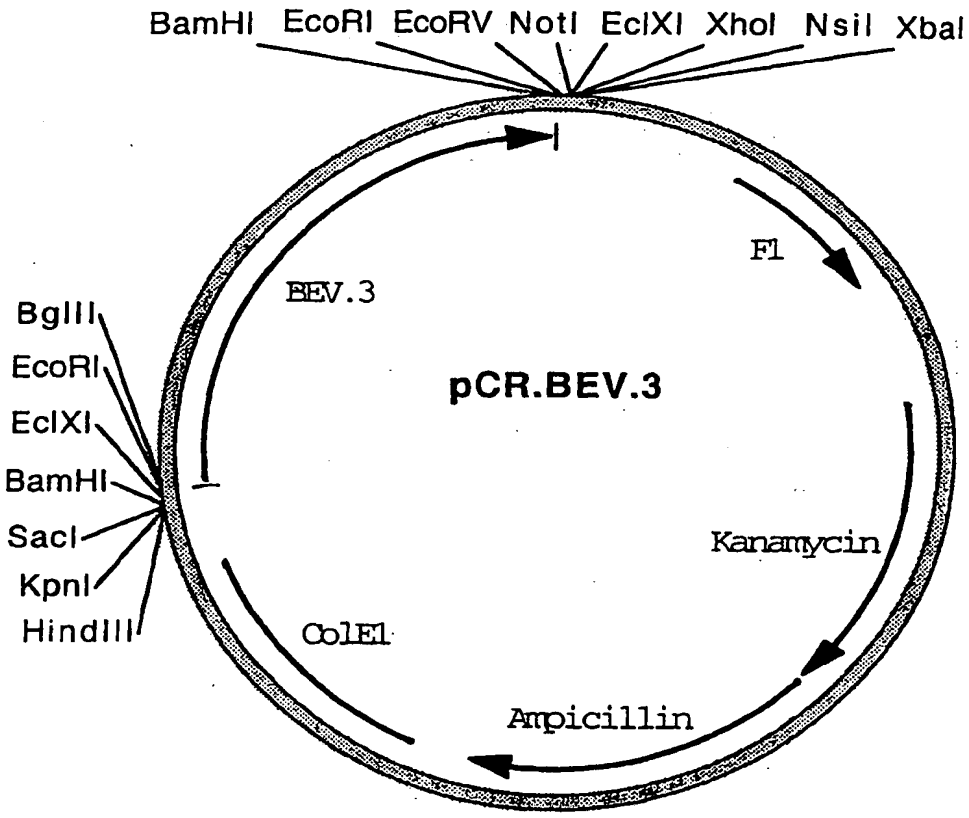

BamHI EcoRI EcoRV NotI EclXI XhoI NsiI XbaI
F1
BEV.3
BglII
EcoRI
EclXI
BamHI
SacI
KpnI
HindIII
pCR.BEV.3
Kanamycin
ColE1
Ampicillin

FIGURE 11

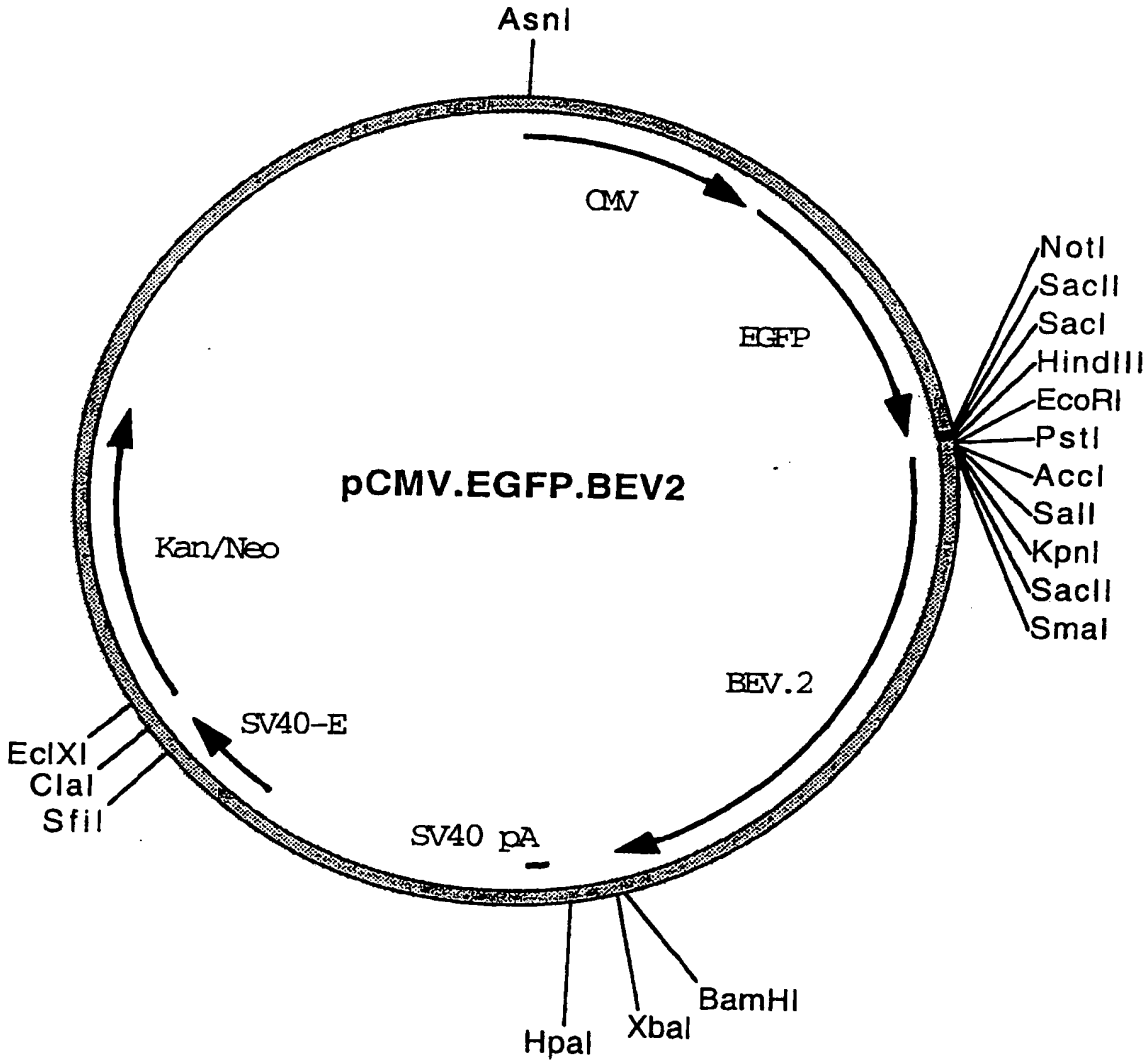

AsnI
CMV
EGFP
pCMV.EGFP.BEV2
Kan/Neo
SV40-E
BEV.2
SV40 pA
NotI
SacII
SacI
HindIII
EcoRI
PstI
AccI
SalI
KpnI
SacII
SmaI
EclXI
ClaI
SfiI
HpaI
XbaI
BamHI

FIGURE 12

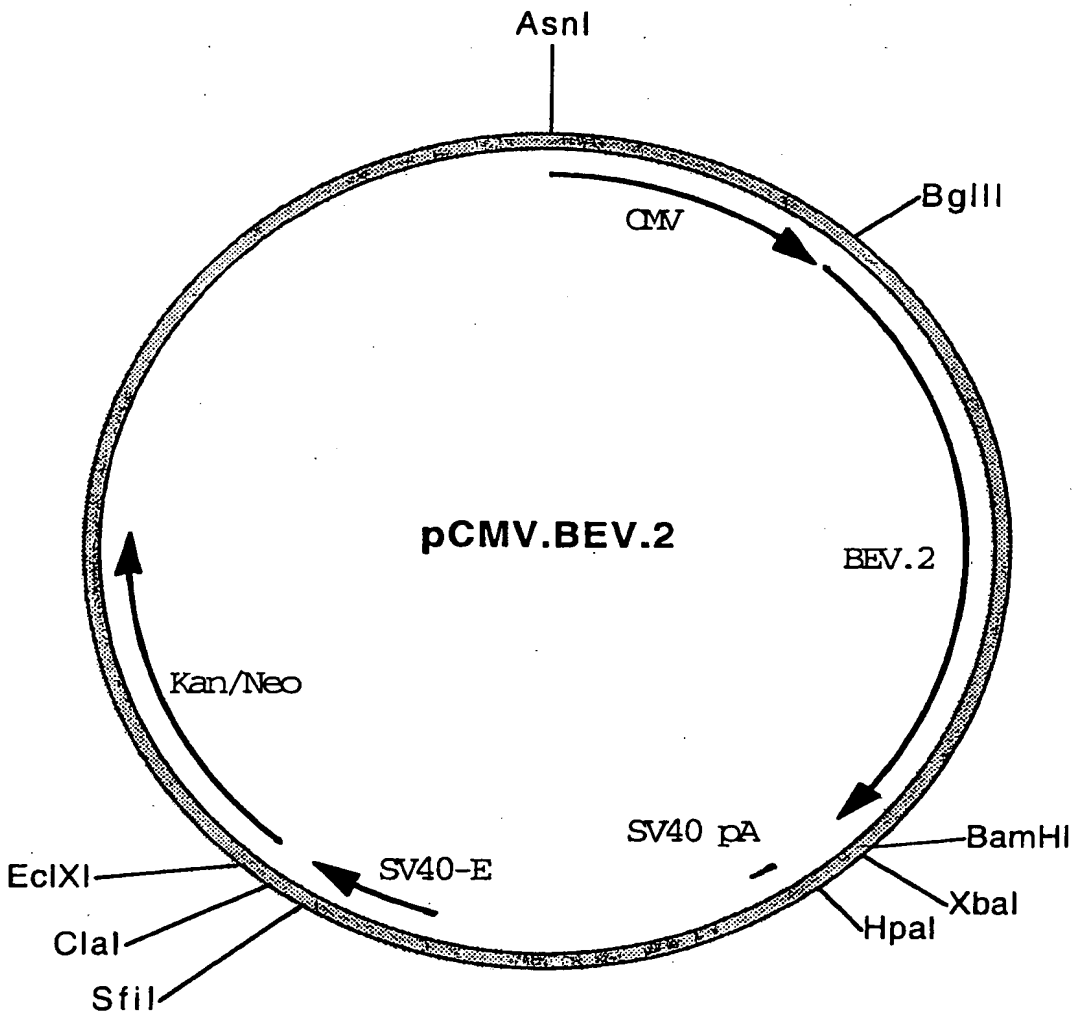

AsnI
BglII
CMV
pCMV.BEV.2
BEV.2
Kan/Neo
SV40 pA
BamHI
SV40-E
EclXI
XbaI
ClaI
HpaI
SfiI

FIGURE 13

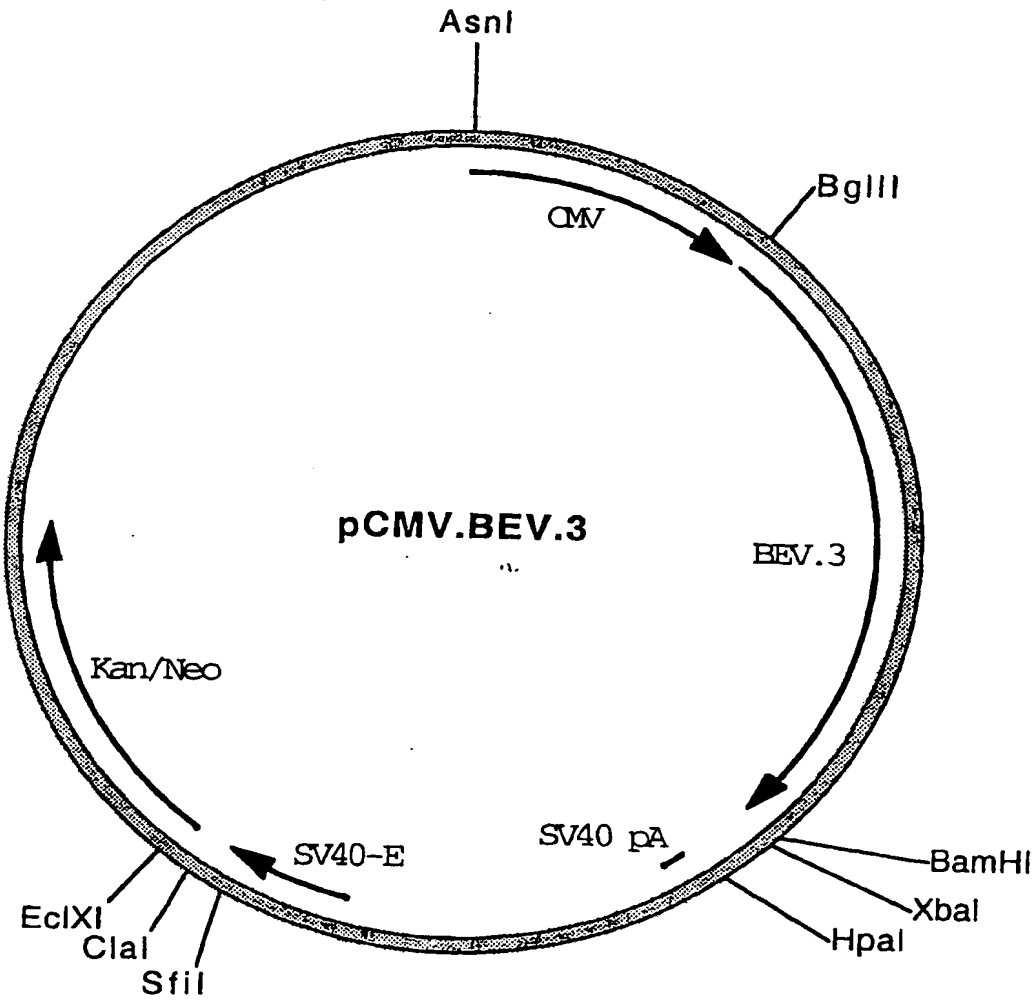

AsnI
BglII
CMV
pCMV.BEV.3
BEV.3
Kan/Neo
SV40-E
SV40 pA
BamHI
XbaI
HpaI
EclXI
ClaI
SfiI

FIGURE 14

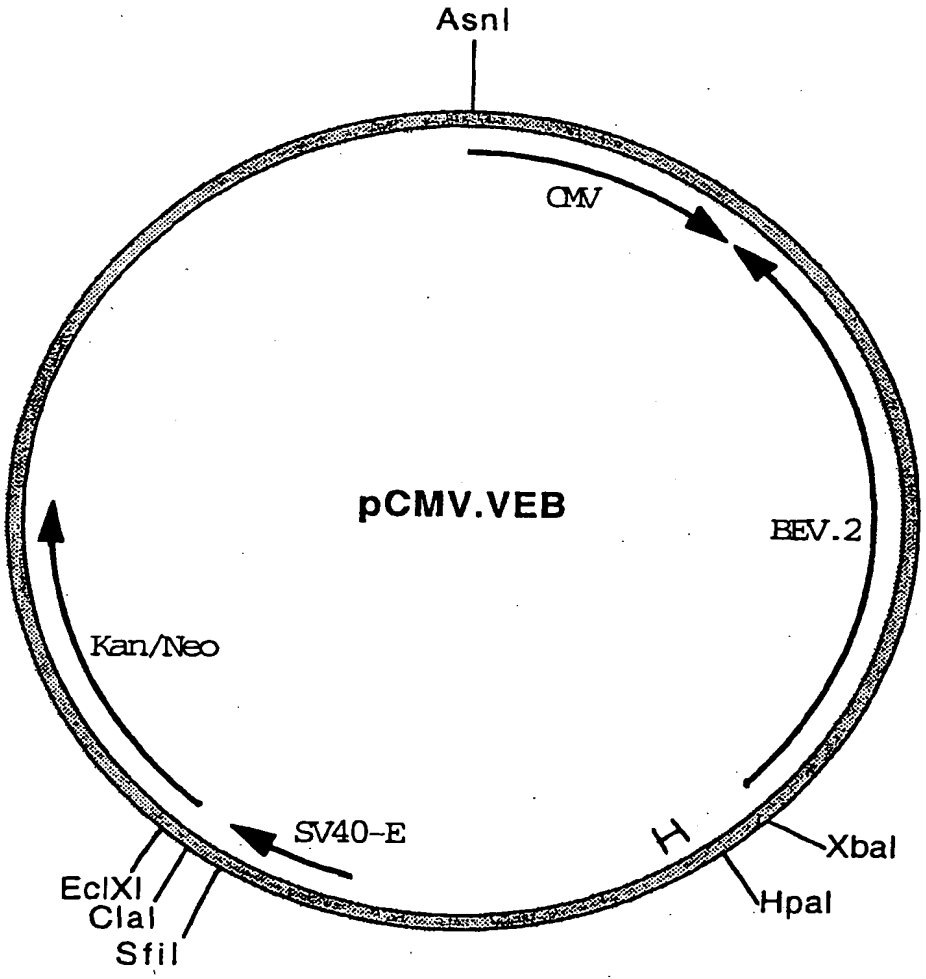

AsnI
CMV
pCMV.VEB
BEV.2
Kan/Neo
SV40-E
H
XbaI
HpaI
EclXI
ClaI
SfiI

FIGURE 15

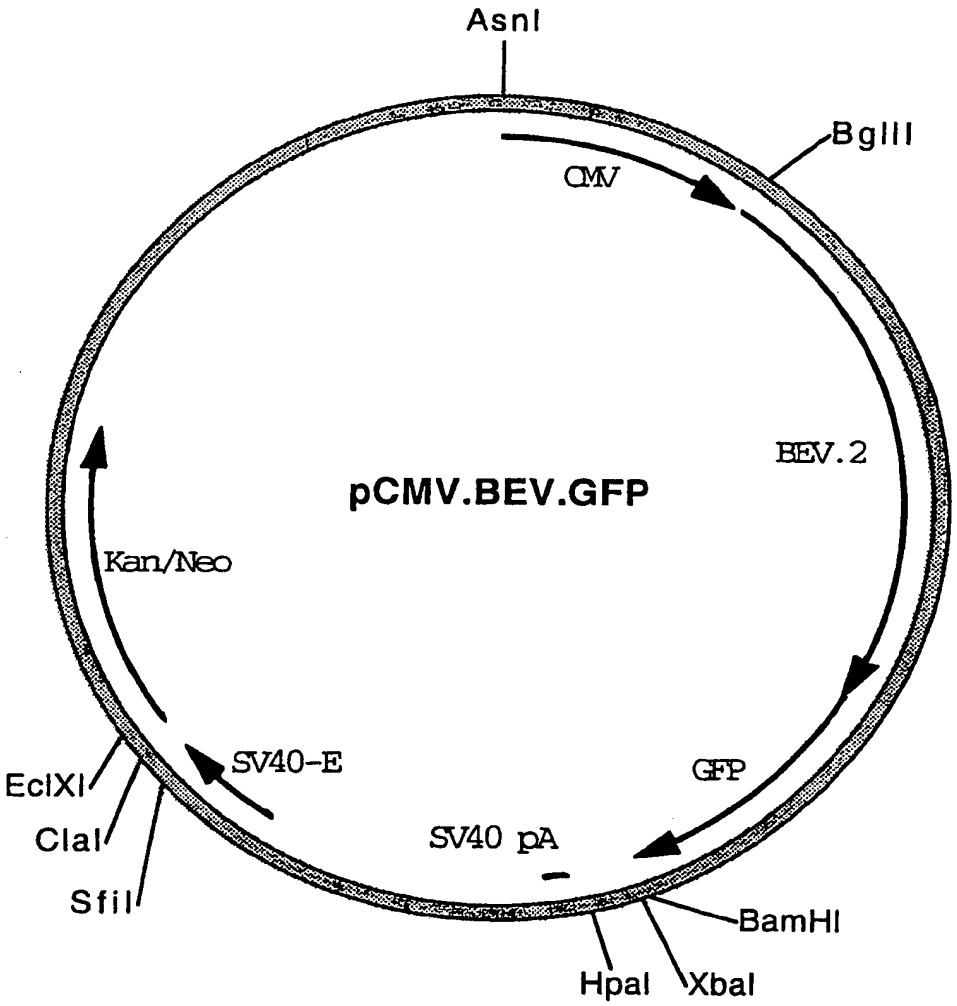

AsnI
BglII
CMV
BEV.2
pCMV.BEV.GFP
Kan/Neo
SV40-E
EclXI
ClaI
SfiI
SV40 pA
GFP
BamHI
HpaI
XbaI

FIGURE 16

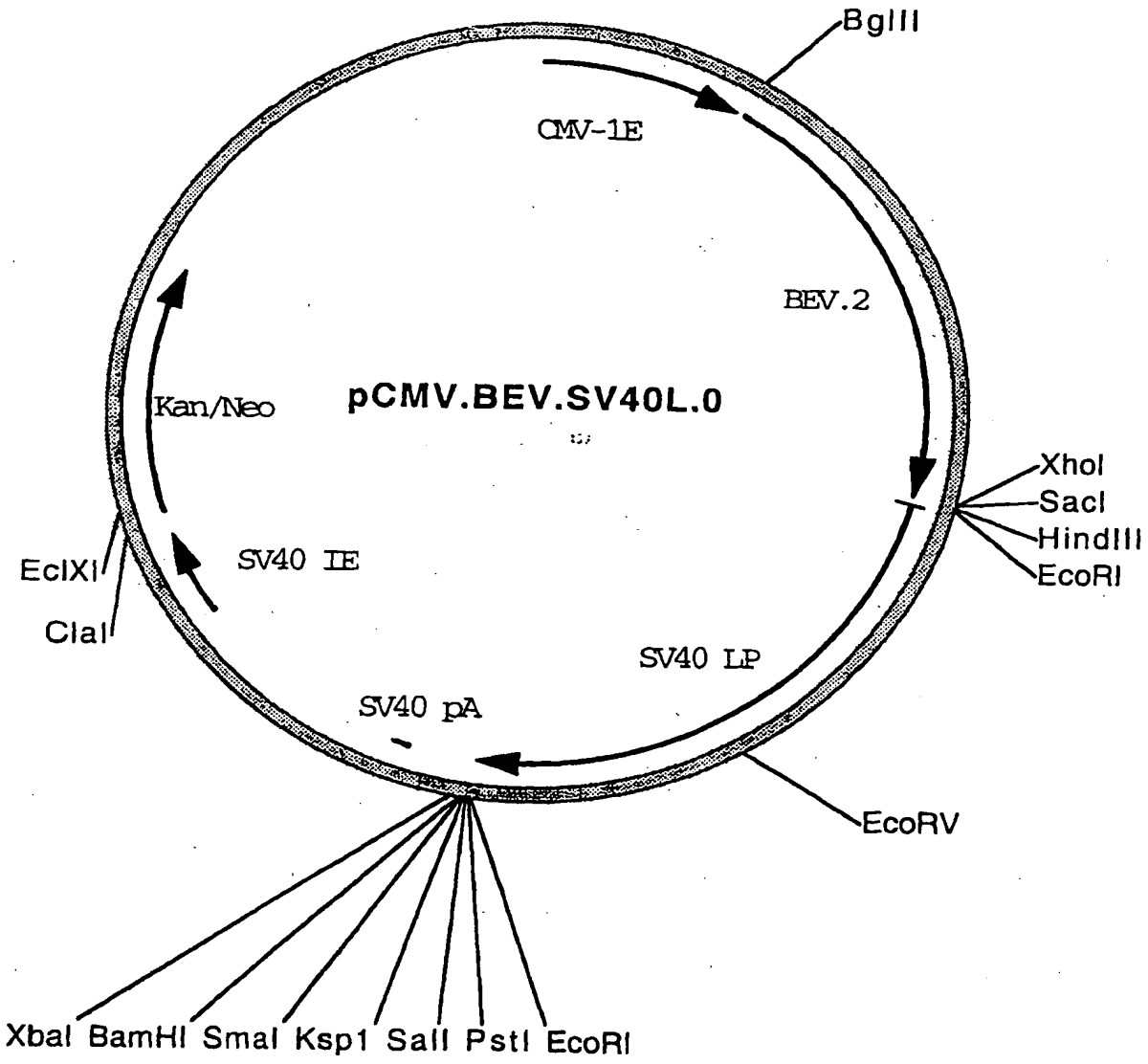

BglII
CMV-1E
BEV.2
pCMV.BEV.SV40L.0
Kan/Neo
XhoI
SacI
HindIII
EcoRI
EclXI
ClaI
SV40 IE
SV40 LP
SV40 pA
EcoRV
XbaI BamHI SmaI Ksp1 SalI PstI EcoRI

FIGURE 17

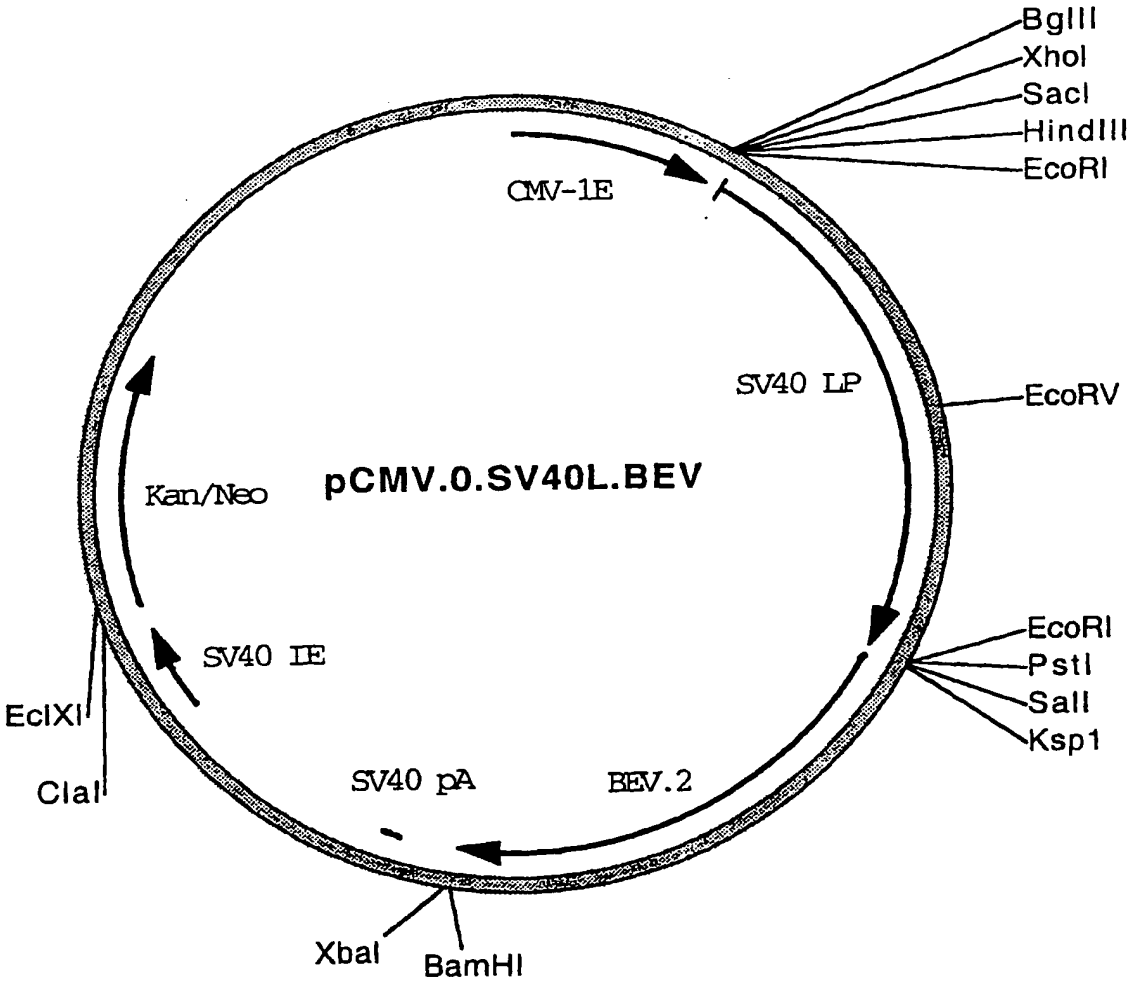

BglII
XhoI
SacI
HindIII
EcoRI
CMV-1E
SV40 LP
EcoRV
Kan/Neo
pCMV.0.SV40L.BEV
SV40 IE
EcoRI
PstI
SalI
Ksp1
EclXI
ClaI
SV40 pA
BEV.2
XbaI
BamHI

FIGURE 18

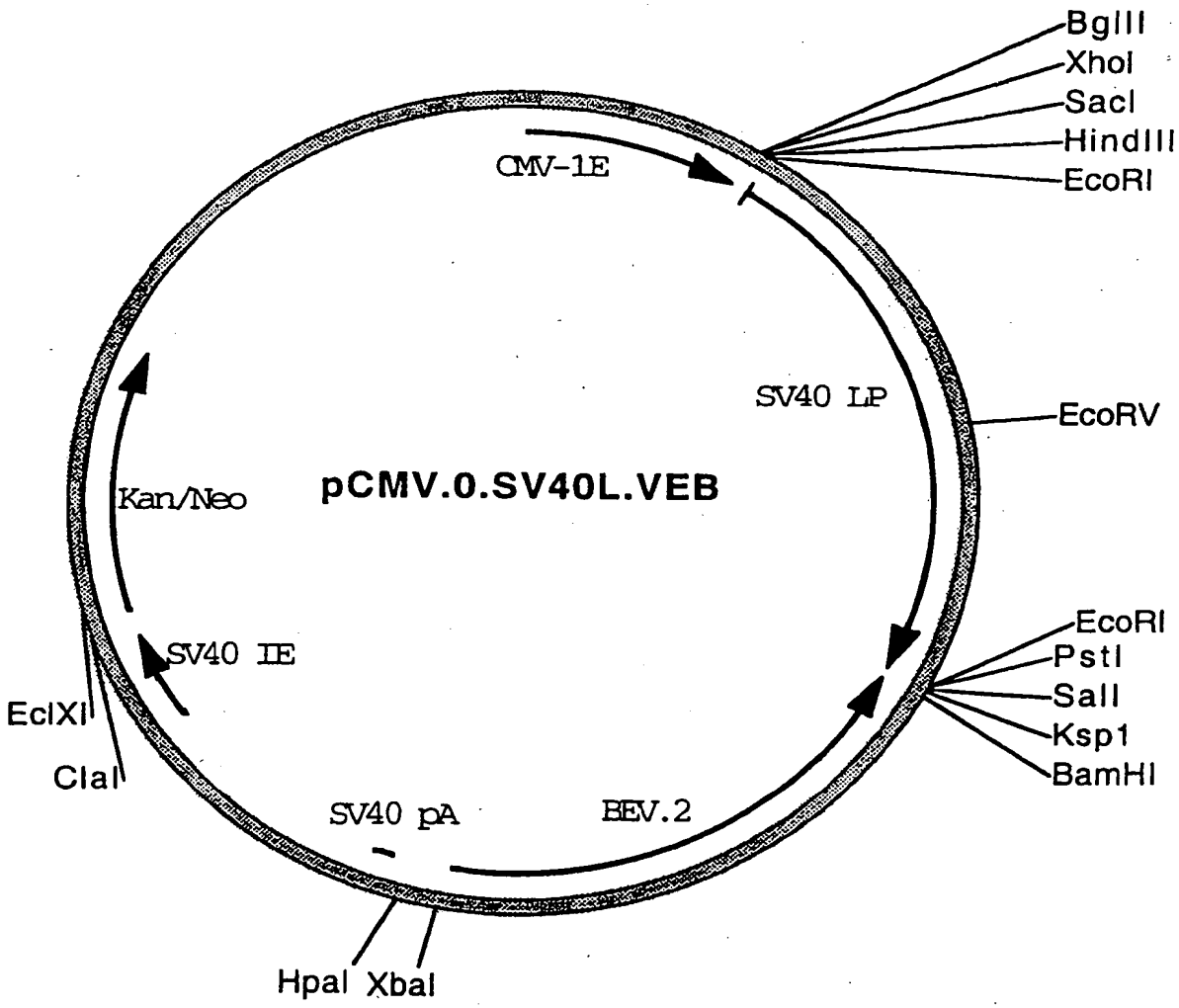

BglII
XhoI
SacI
HindIII
EcoRI
CMV-1E
SV40 LP
EcoRV
Kan/Neo
pCMV.0.SV40L.VEB
SV40 IE
EcoRI
PstI
SalI
Ksp1
BamHI
EclXI
ClaI
SV40 pA
BEV.2
HpaI
XbaI

FIGURE 19

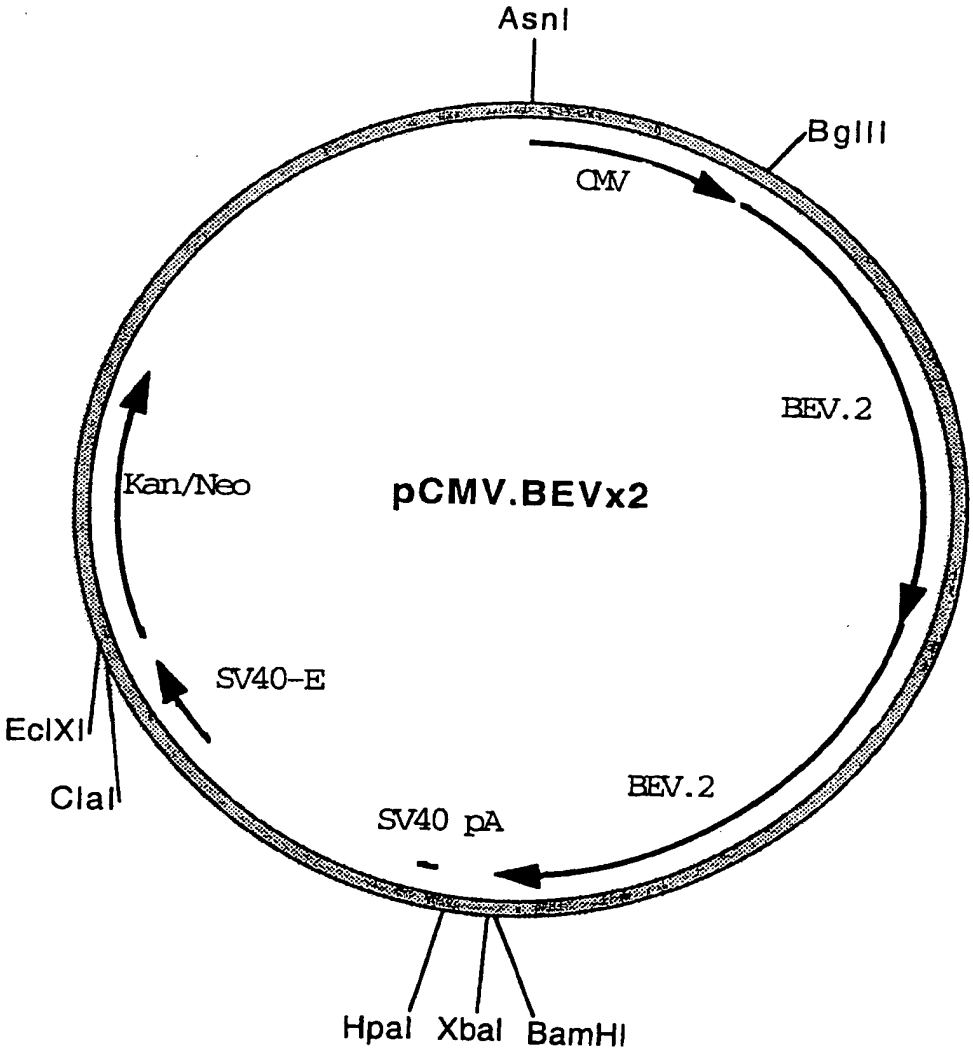

AsnI
BglII
CMV
BEV.2
Kan/Neo
pCMV.BEVx2
SV40-E
EclXI
ClaI
BEV.2
SV40 pA
HpaI
XbaI
BamHI

FIGURE 20

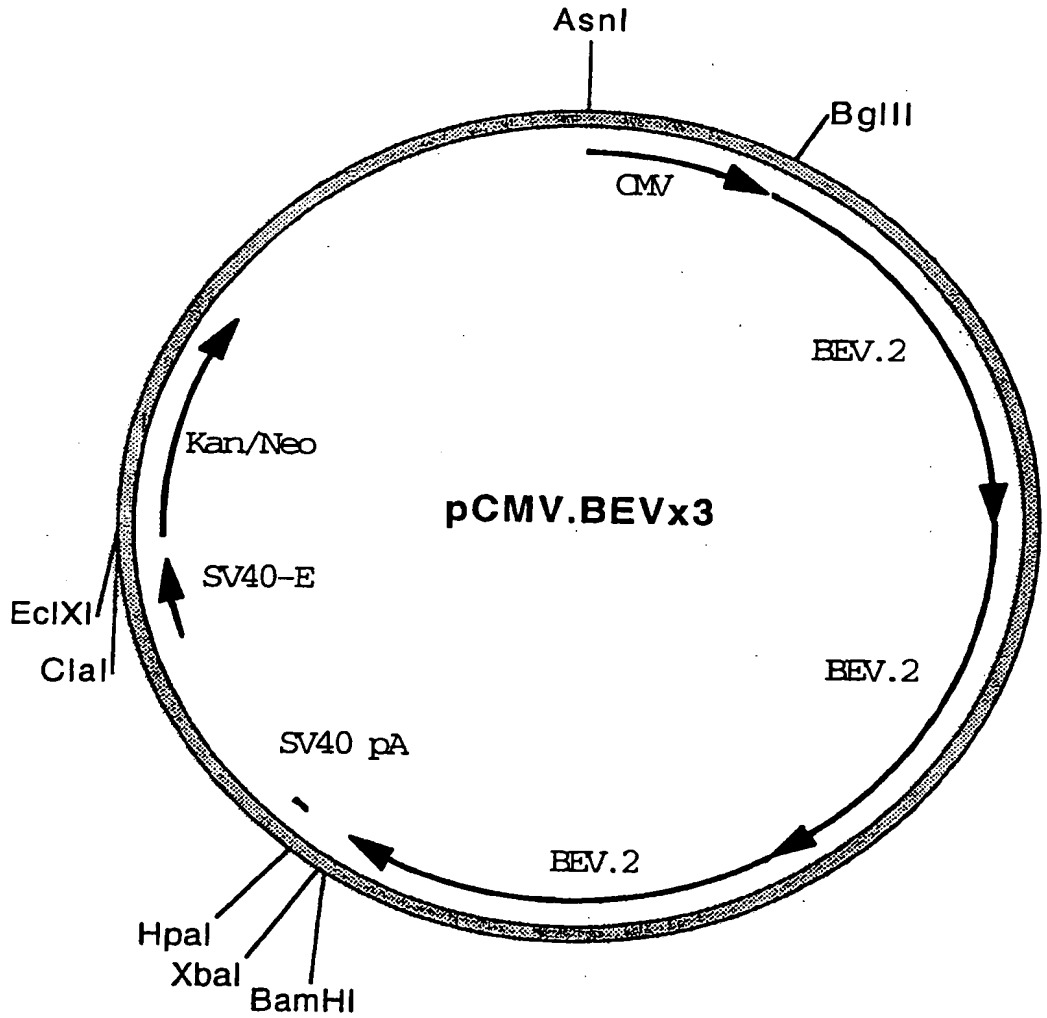

AsnI
BglII
CMV
BEV.2
Kan/Neo
pCMV.BEVx3
SV40-E
EclXI
ClaI
BEV.2
SV40 pA
BEV.2
HpaI
XbaI
BamHI

FIGURE 21

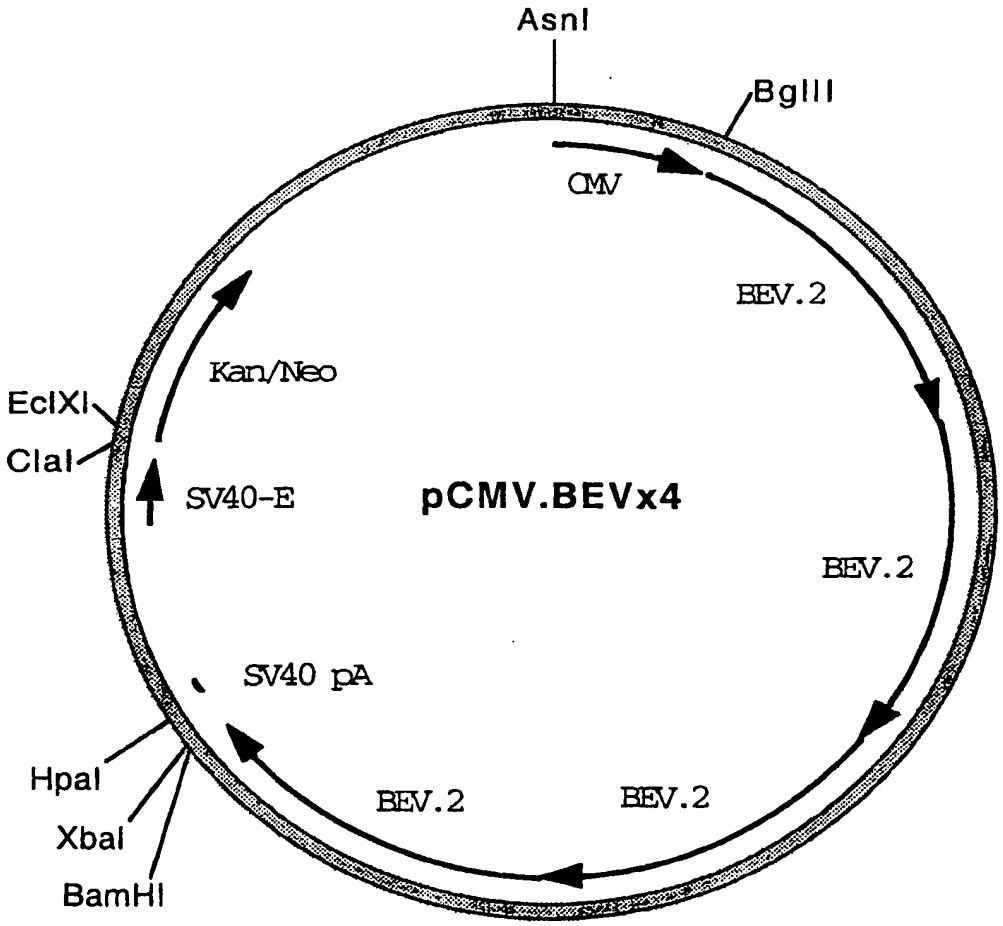

AsnI
BglII
CMV
BEV.2
Kan/Neo
EclXI
ClaI
SV40-E
pCMV.BEVx4
BEV.2
SV40 pA
HpaI
XbaI
BamHI
BEV.2
BEV.2

FIGURE 22

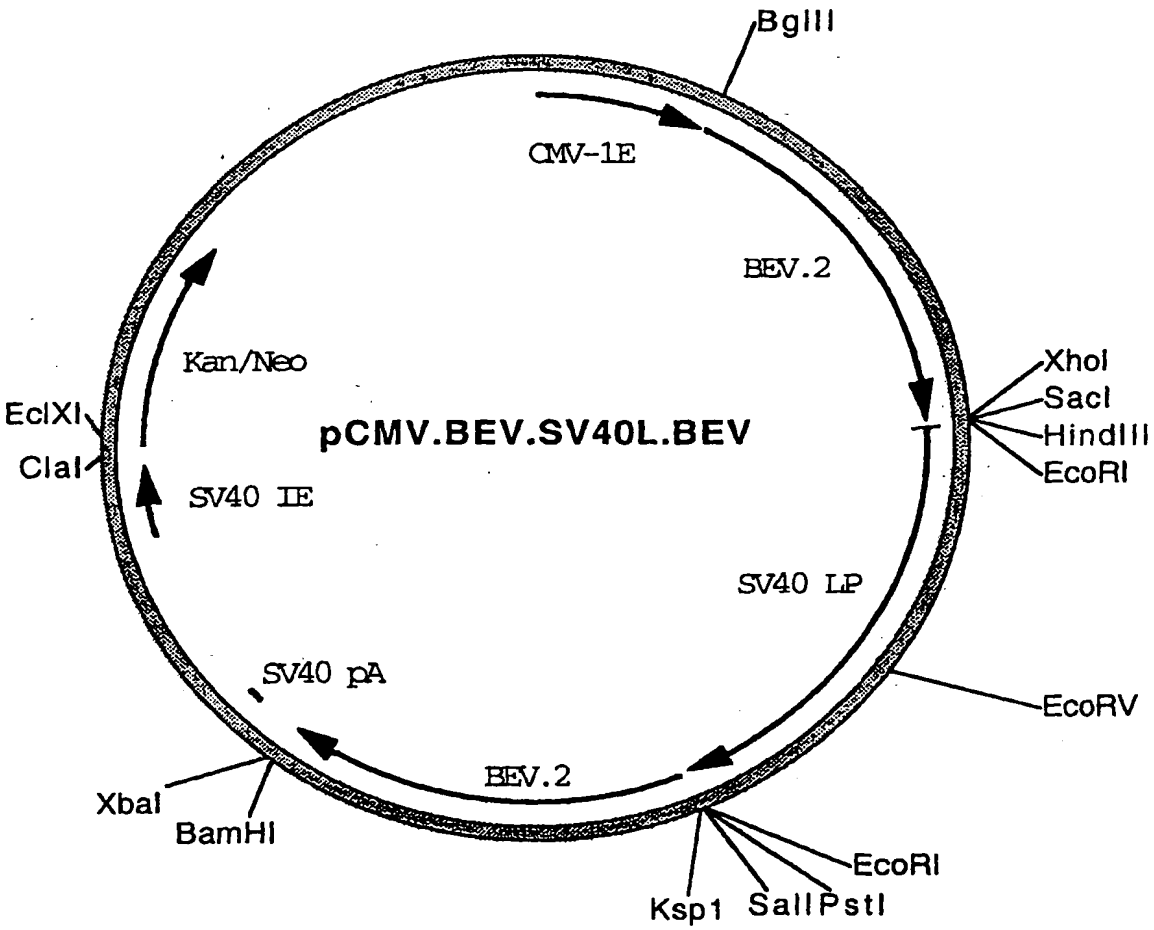

BglII
CMV-1E
BEV.2
Kan/Neo
EclXI
ClaI
pCMV.BEV.SV40L.BEV
SV40 IE
XhoI
SacI
HindIII
EcoRI
SV40 LP
SV40 pA
EcoRV
BEV.2
XbaI
BamHI
EcoRI
Ksp1
SalI
PstI

FIGURE 23

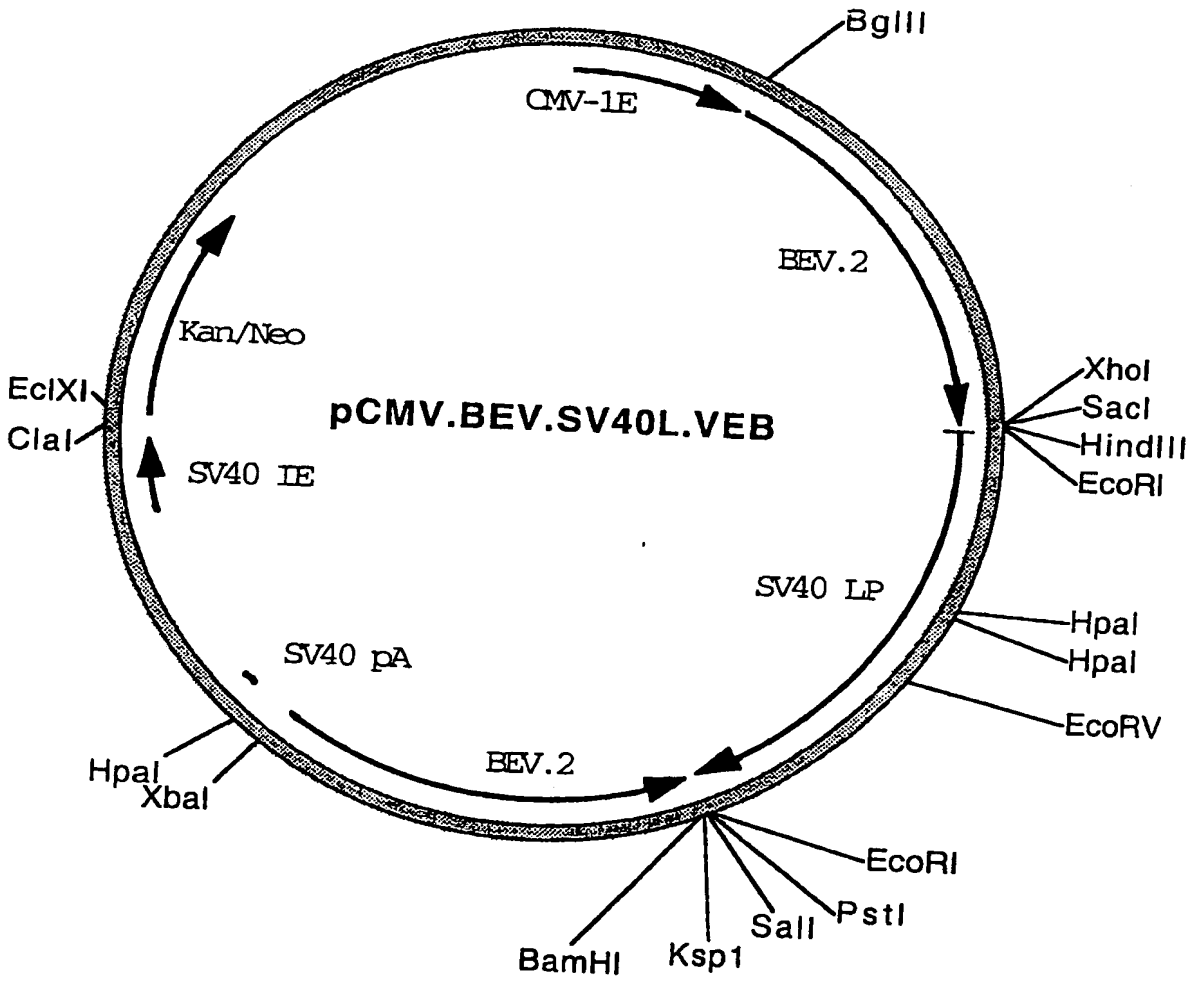

BglII
CMV-1E
BEV.2
Kan/Neo
EclXI
ClaI
pCMV.BEV.SV40L.VEB
SV40 IE
XhoI
SacI
HindIII
EcoRI
SV40 LP
HpaI
HpaI
EcoRV
SV40 pA
HpaI
XbaI
BEV.2
EcoRI
PstI
SalI
BamHI
Ksp1

FIGURE 24

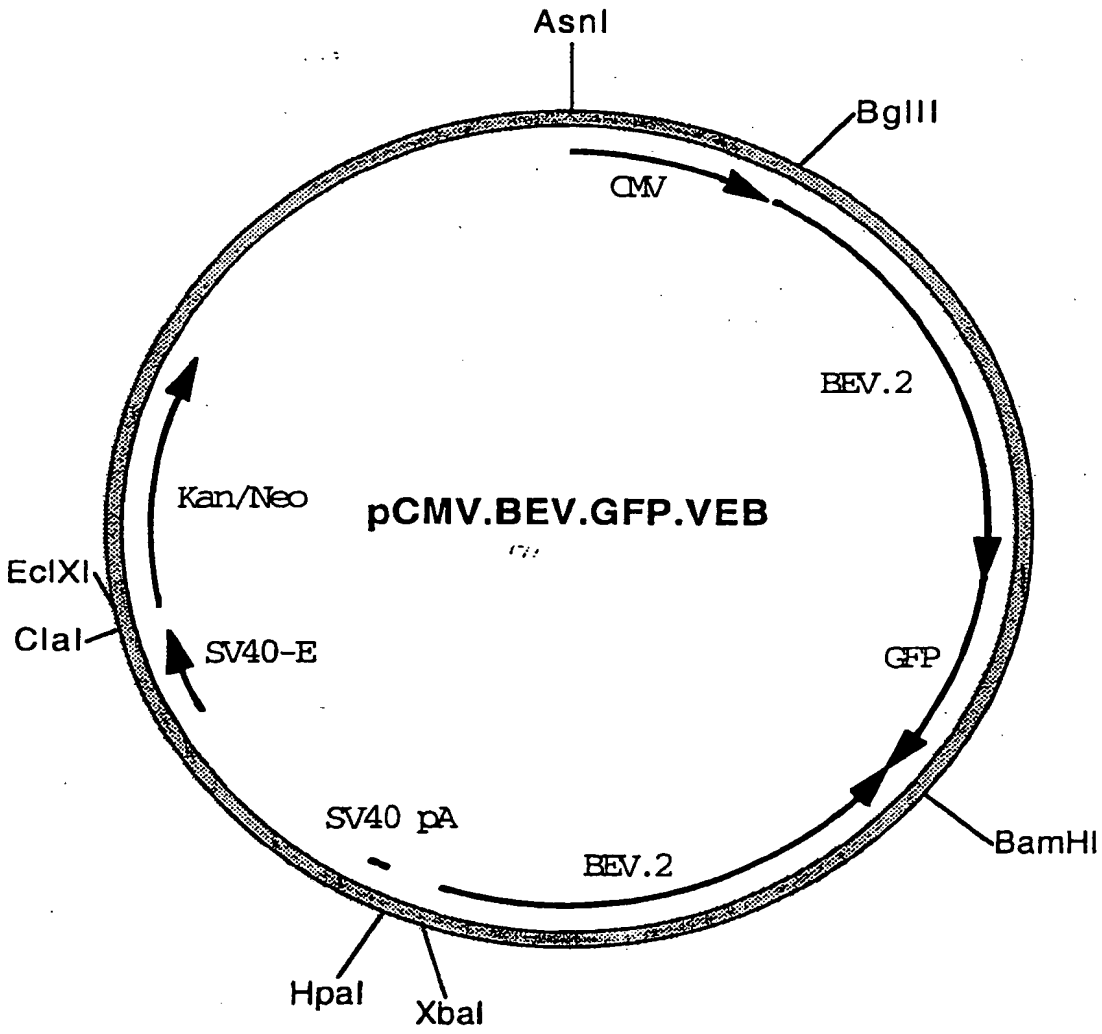

AsnI
BglII
CMV
BEV.2
Kan/Neo
pCMV.BEV.GFP.VEB
EclXI
ClaI
SV40-E
GFP
SV40 pA
BamHI
BEV.2
HpaI
XbaI

FIGURE 25

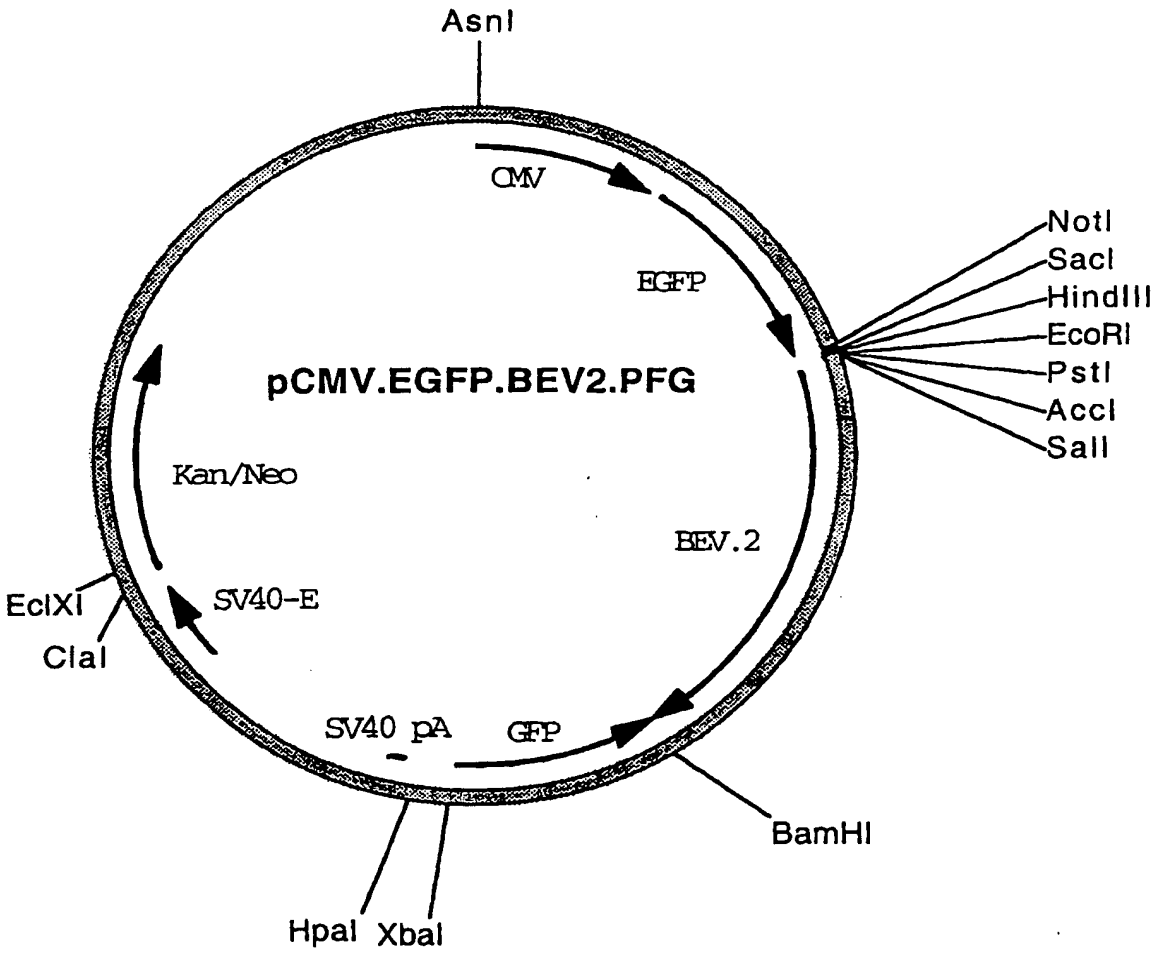

AsnI
CMV
EGFP
NotI
SacI
HindIII
EcoRI
PstI
AccI
SalI
pCMV.EGFP.BEV2.PFG
Kan/Neo
BEV.2
EclXI
ClaI
SV40-E
SV40 pA
GFP
BamHI
HpaI
XbaI

FIGURE 26

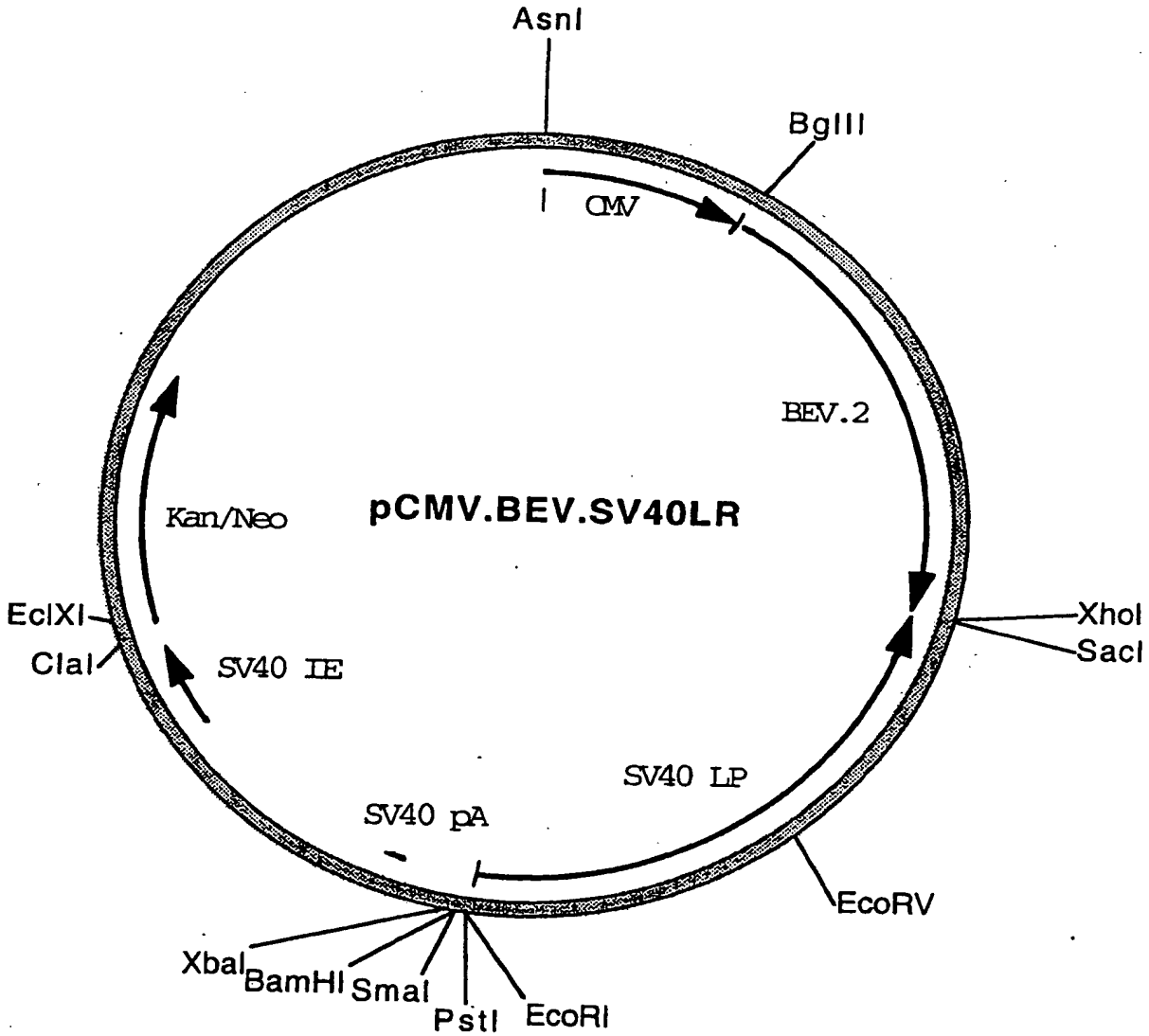

AsnI
BglII
CMV
BEV.2
pCMV.BEV.SV40LR
Kan/Neo
EclXI
ClaI
SV40 IE
XhoI
SacI
SV40 LP
SV40 pA
EcoRV
XbaI
BamHI
SmaI
PstI
EcoRI

FIGURE 27

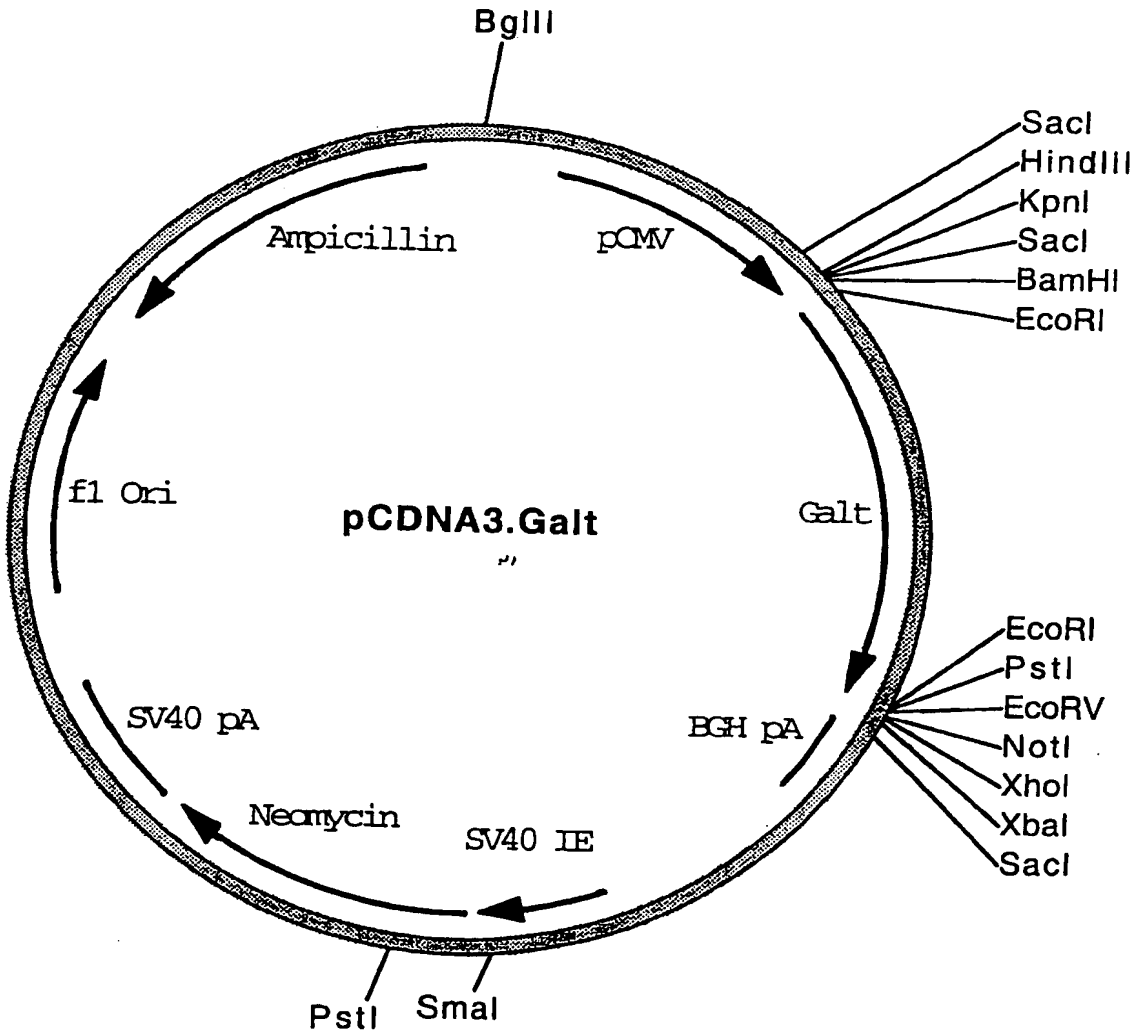

BglII
SacI
HindIII
KpnI
SacI
BamHI
EcoRI
Ampicillin
pCMV
f1 Ori
pCDNA3.Galt
Galt
EcoRI
PstI
EcoRV
NotI
XhoI
XbaI
SacI
SV40 pA
BGH pA
Neomycin
SV40 IE
PstI
SmaI

FIGURE 28

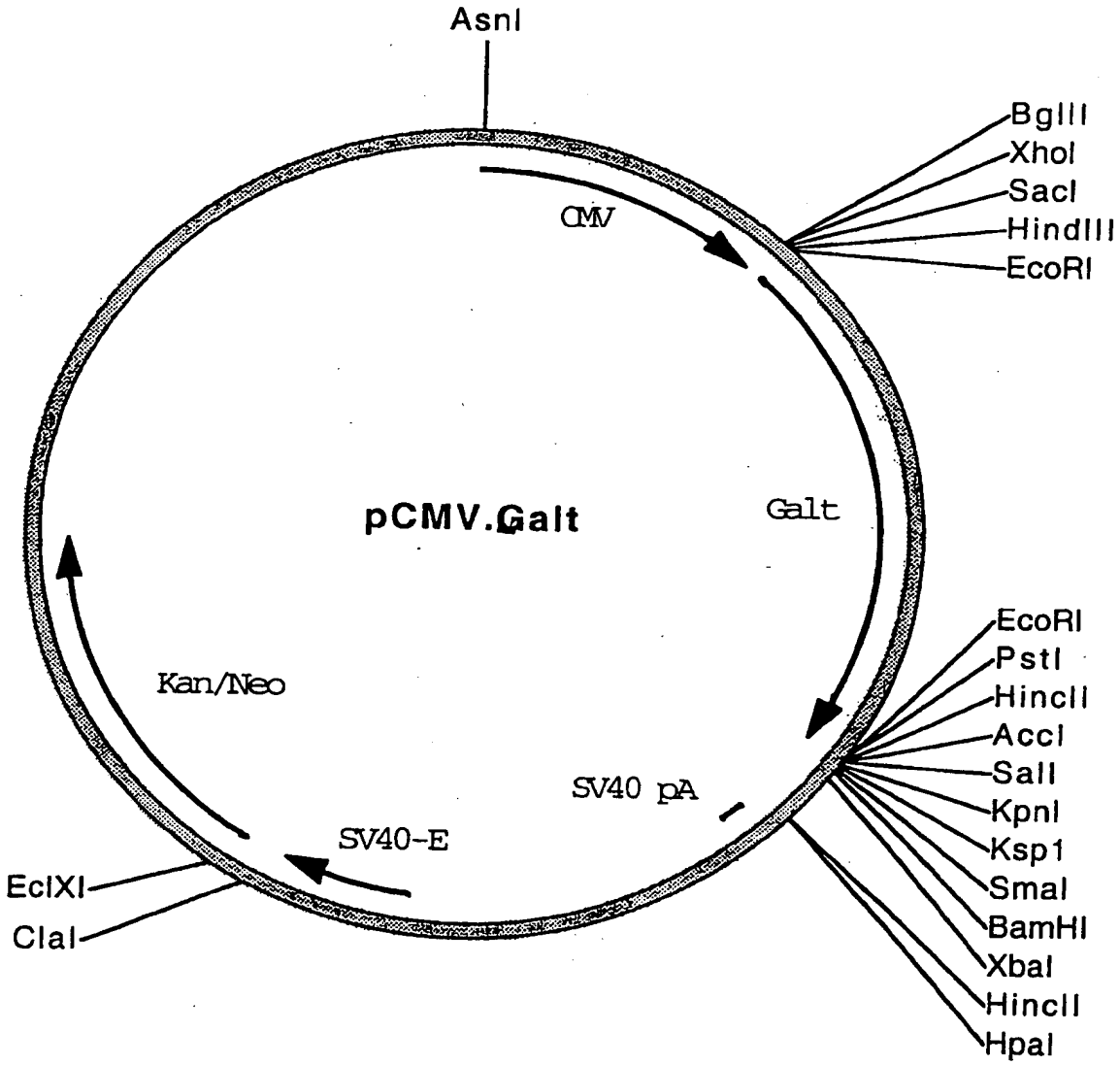

AsnI
BglII
XhoI
SacI
HindIII
EcoRI
CMV
pCMV.Galt
Galt
EcoRI
PstI
HincII
AccI
SalI
KpnI
Ksp1
SmaI
BamHI
XbaI
HincII
HpaI
SV40 pA
SV40-E
Kan/Neo
EclXI
ClaI

FIGURE 29

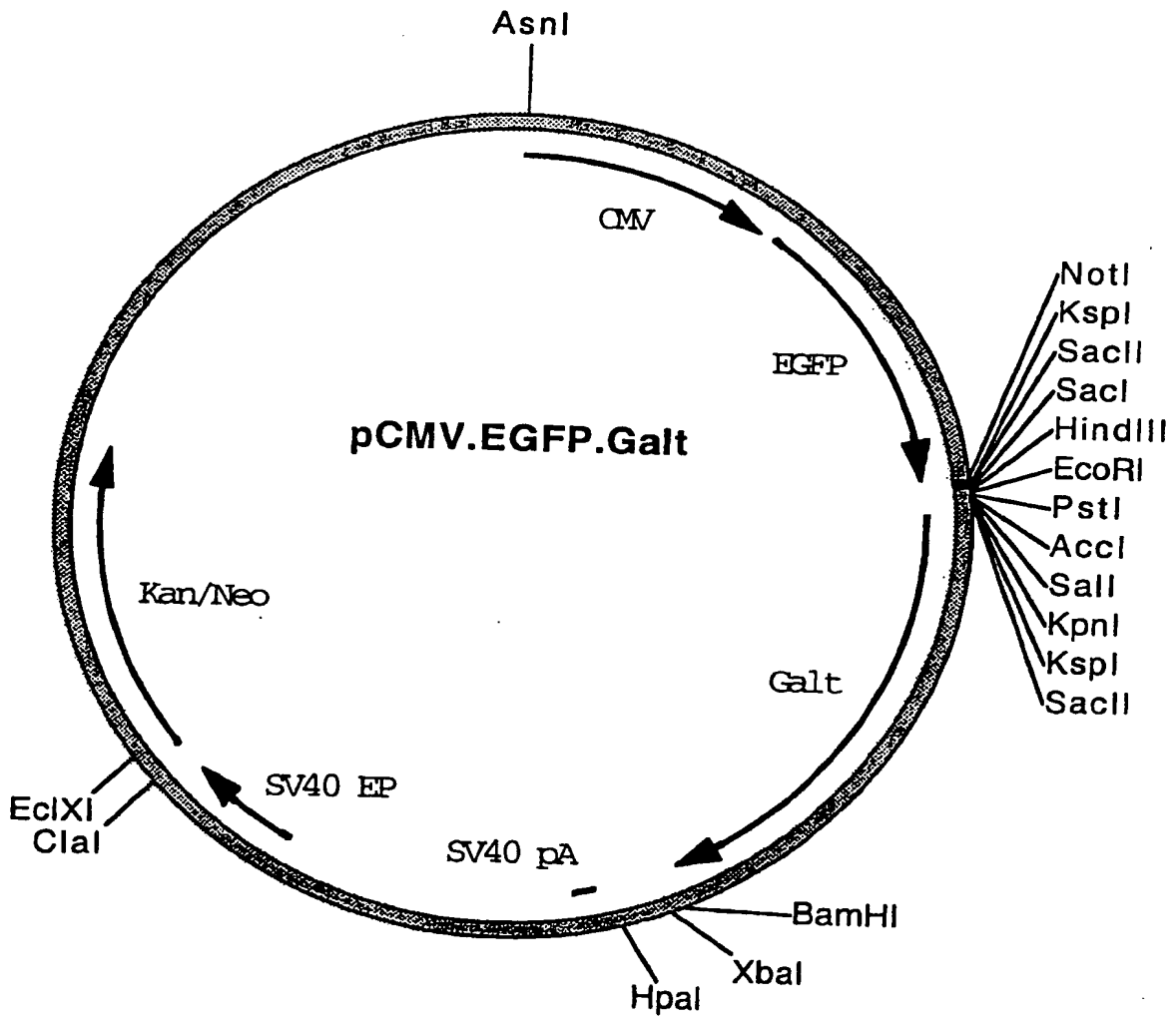

AsnI
CMV
EGFP
pCMV.EGFP.GalT
Kan/Neo
GalT
SV40 EP
SV40 pA
EclXI
ClaI
BamHI
XbaI
HpaI
NotI
KspI
SacII
SacI
HindIII
EcoRI
PstI
AccI
SalI
KpnI
KspI
SacII

FIGURE 30

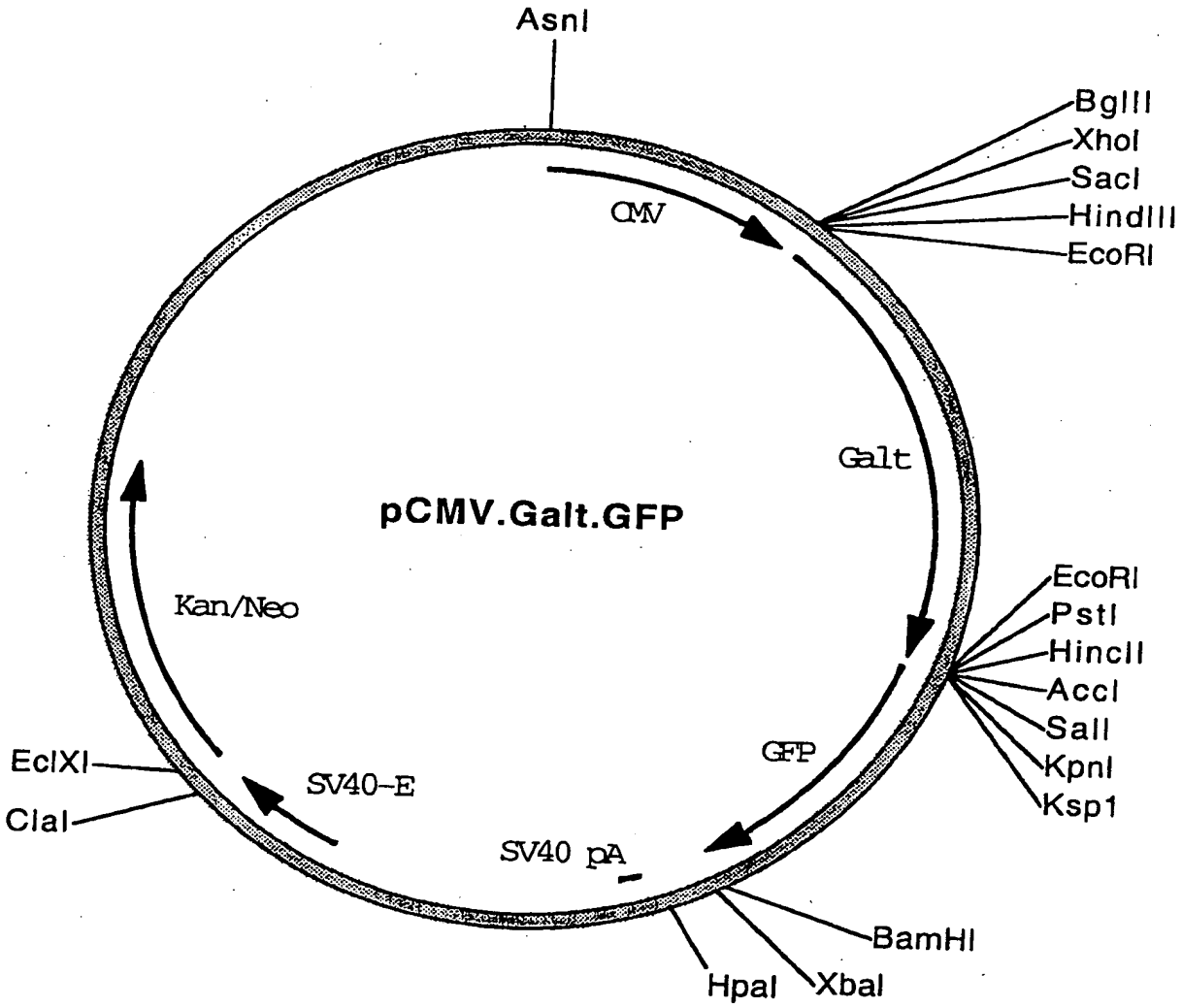

AsnI
BglII
XhoI
SacI
HindIII
EcoRI
CMV
Galt
pCMV.Galt.GFP
Kan/Neo
EcoRI
PstI
HincII
AccI
SalI
KpnI
Ksp1
EclXI
ClaI
SV40-E
GFP
SV40 pA
BamHI
HpaI
XbaI

FIGURE 31

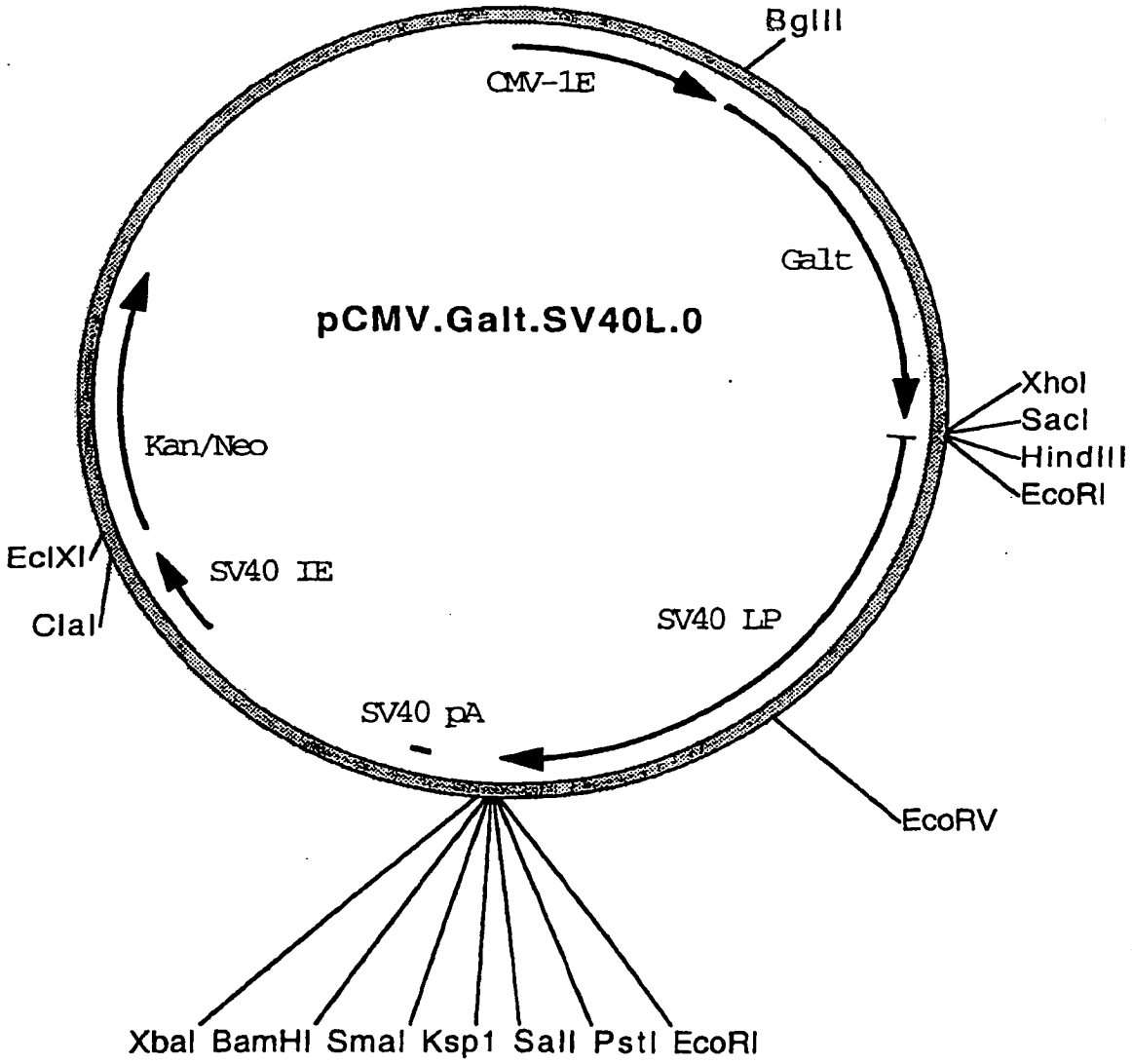

BglII
CMV-1E
Galt
pCMV.Galt.SV40L.0
XhoI
SacI
HindIII
EcoRI
Kan/Neo
EclXI
ClaI
SV40 IE
SV40 LP
SV40 pA
EcoRV
XbaI BamHI SmaI Ksp1 SalI PstI EcoRI

FIGURE 32

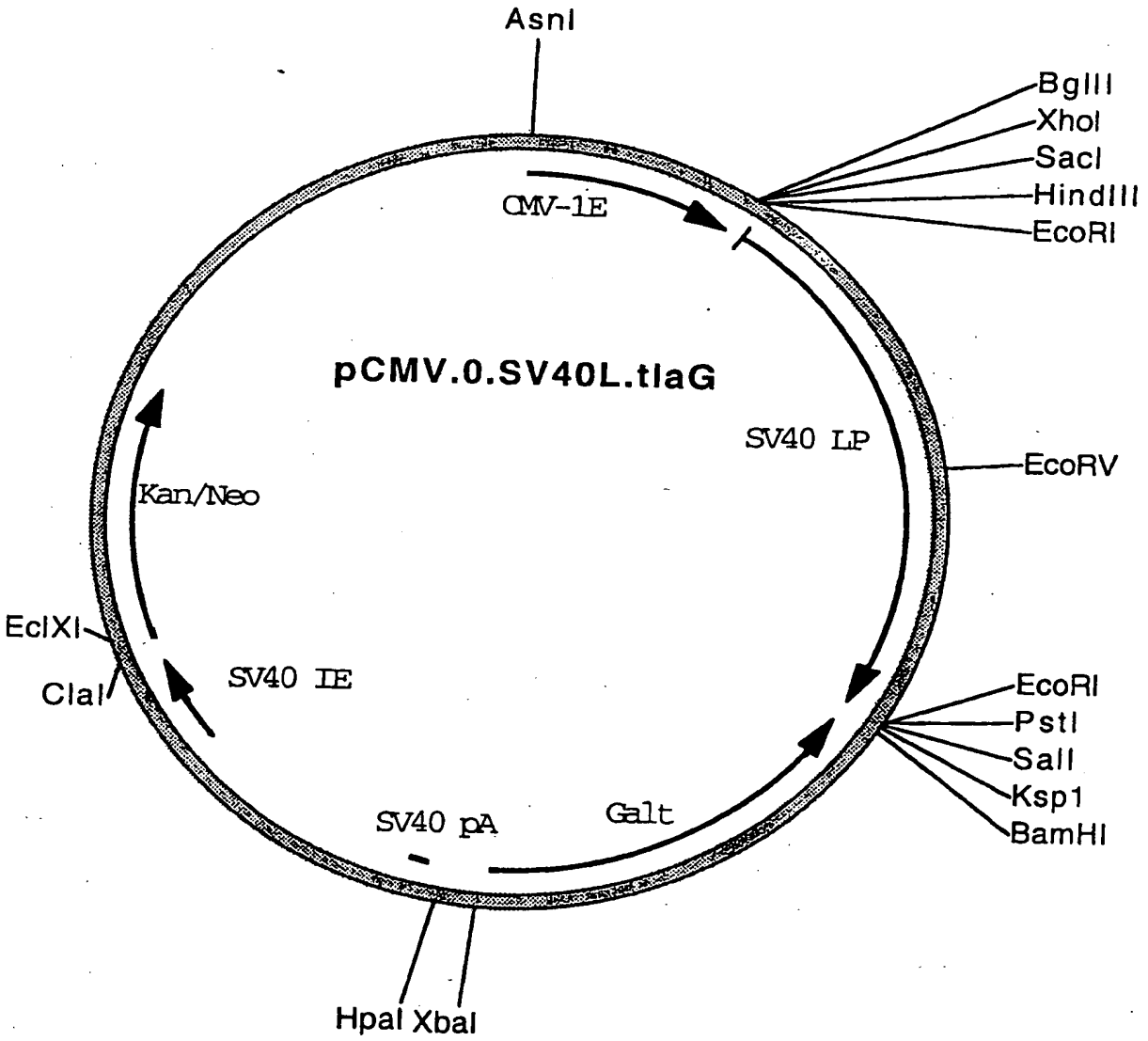

AsnI
BglII
XhoI
SacI
HindIII
EcoRI
CMV-1E
pCMV.0.SV40L.tlaG
SV40 LP
EcoRV
Kan/Neo
EclXI
ClaI
SV40 IE
EcoRI
PstI
SalI
Ksp1
BamHI
SV40 pA
Galt
HpaI
XbaI

FIGURE 33

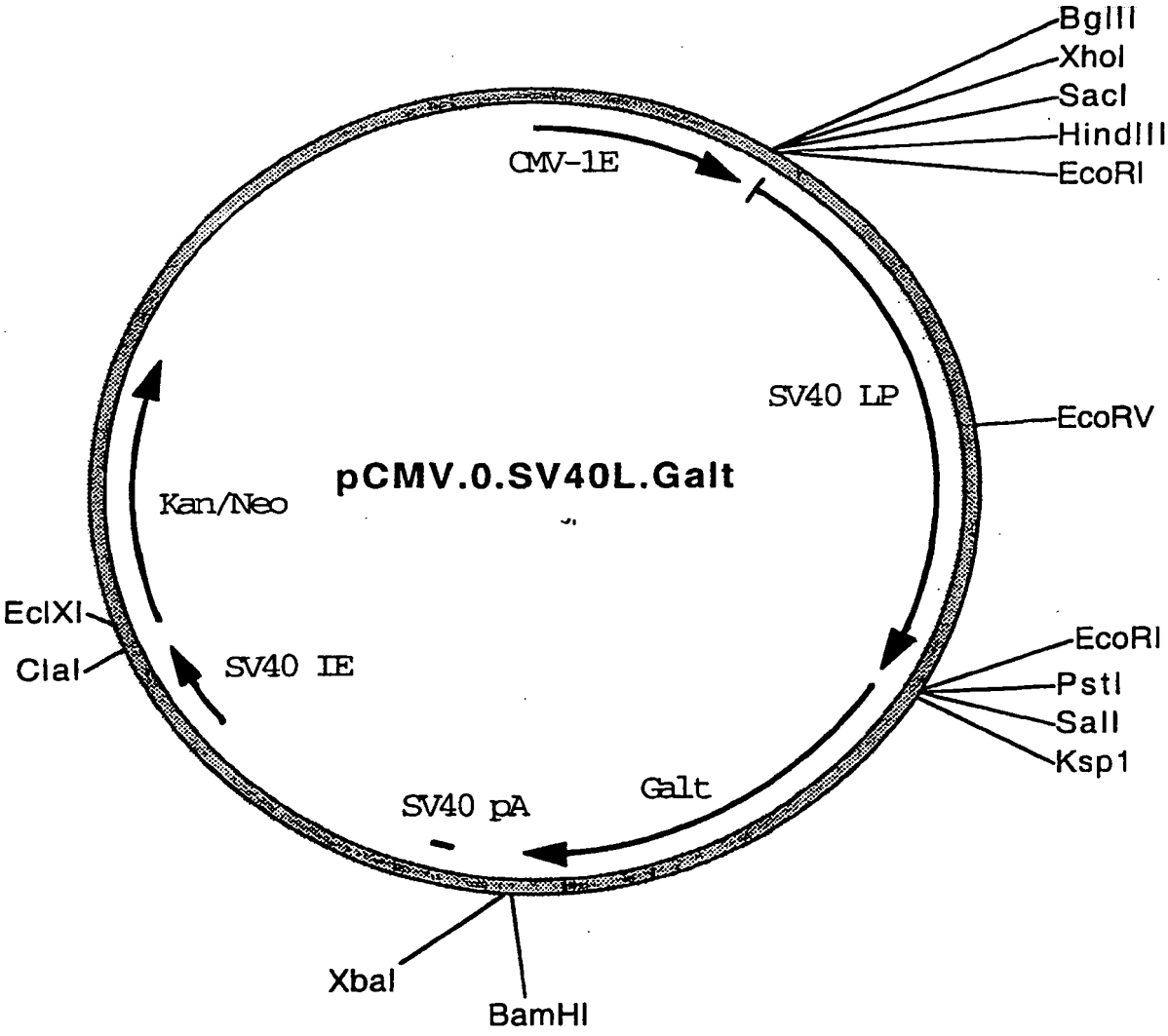

BglII
XhoI
SacI
HindIII
EcoRI
CMV-1E
SV40 LP
EcoRV
pCMV.0.SV40L.Galt
Kan/Neo
EclXI
ClaI
SV40 IE
EcoRI
PstI
SalI
Ksp1
SV40 pA
Galt
XbaI
BamHI

FIGURE 34

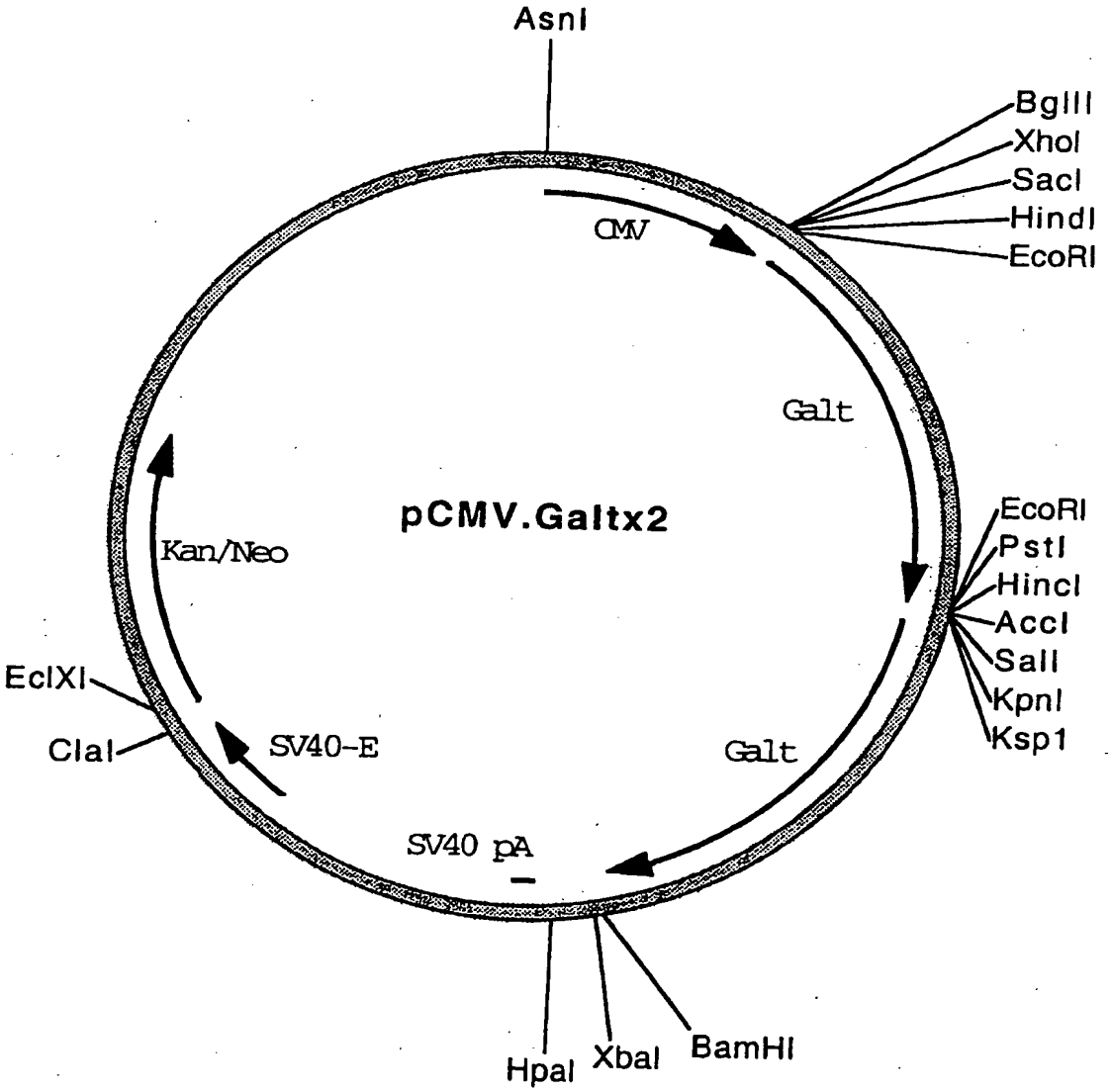

AsnI
BglII
XhoI
SacI
HindI
EcoRI
CMV
Galt
pCMV.Galtx2
Kan/Neo
EcoRI
PstI
HincI
AccI
SalI
KpnI
Ksp1
EclXI
ClaI
SV40-E
Galt
SV40 pA
HpaI
XbaI
BamHI

FIGURE 35

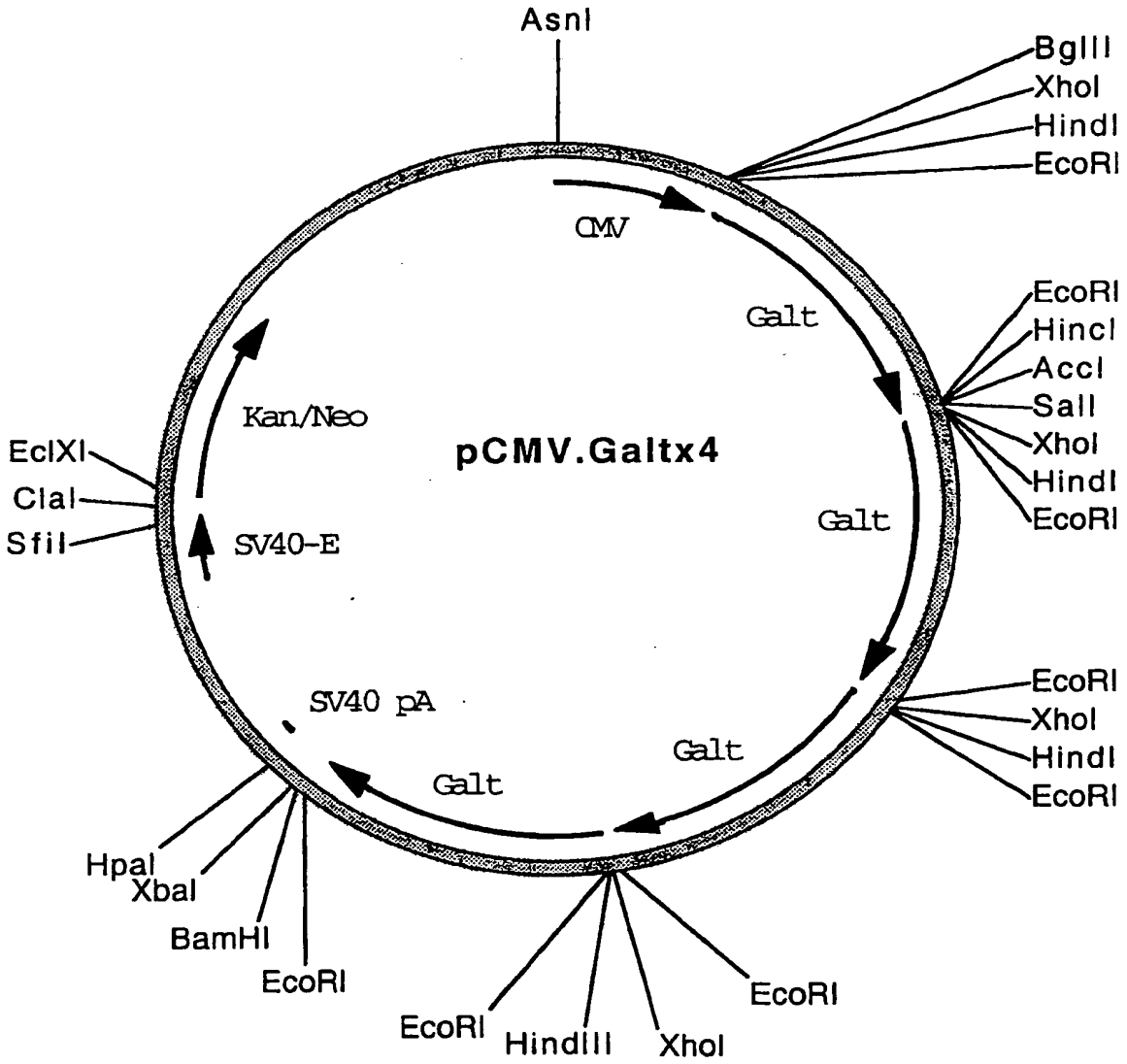

AsnI
BglII
XhoI
HindI
EcoRI
CMV
Galt
EcoRI
HincI
AccI
SalI
XhoI
HindI
EcoRI
Kan/Neo
pCMV.Galtx4
EclXI
ClaI
SfiI
SV40-E
Galt
EcoRI
XhoI
HindI
EcoRI
SV40 pA
Galt
Galt
HpaI
XbaI
BamHI
EcoRI
EcoRI
HindIII
XhoI
EcoRI

FIGURE 36

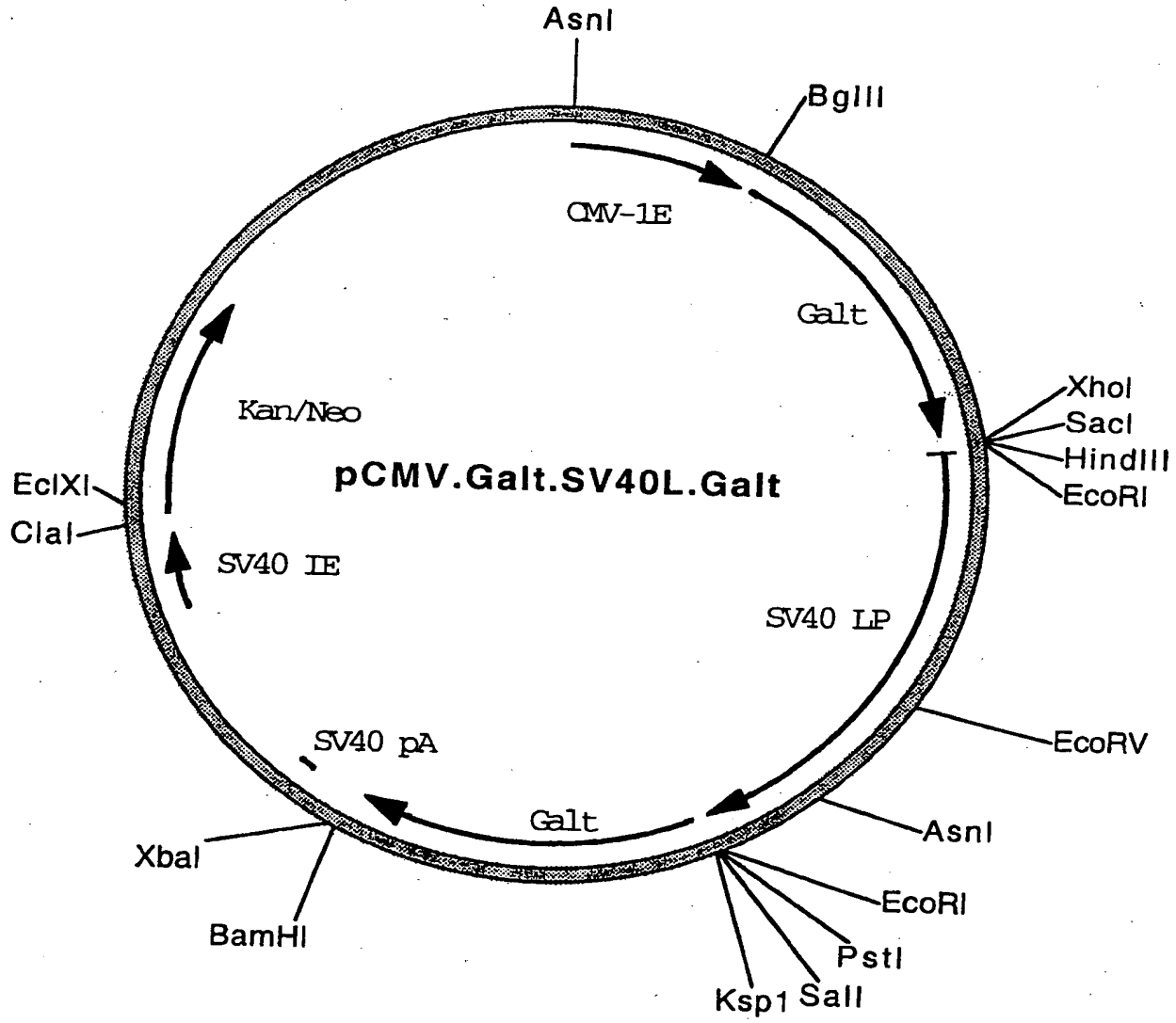

AsnI
BglII
CMV-1E
GalT
XhoI
SacI
HindIII
EcoRI
Kan/Neo
pCMV.GalT.SV40L.GalT
EclXI
ClaI
SV40 IE
SV40 LP
EcoRV
SV40 pA
GalT
AsnI
XbaI
EcoRI
BamHI
PstI
Ksp1 SalI

FIGURE 37

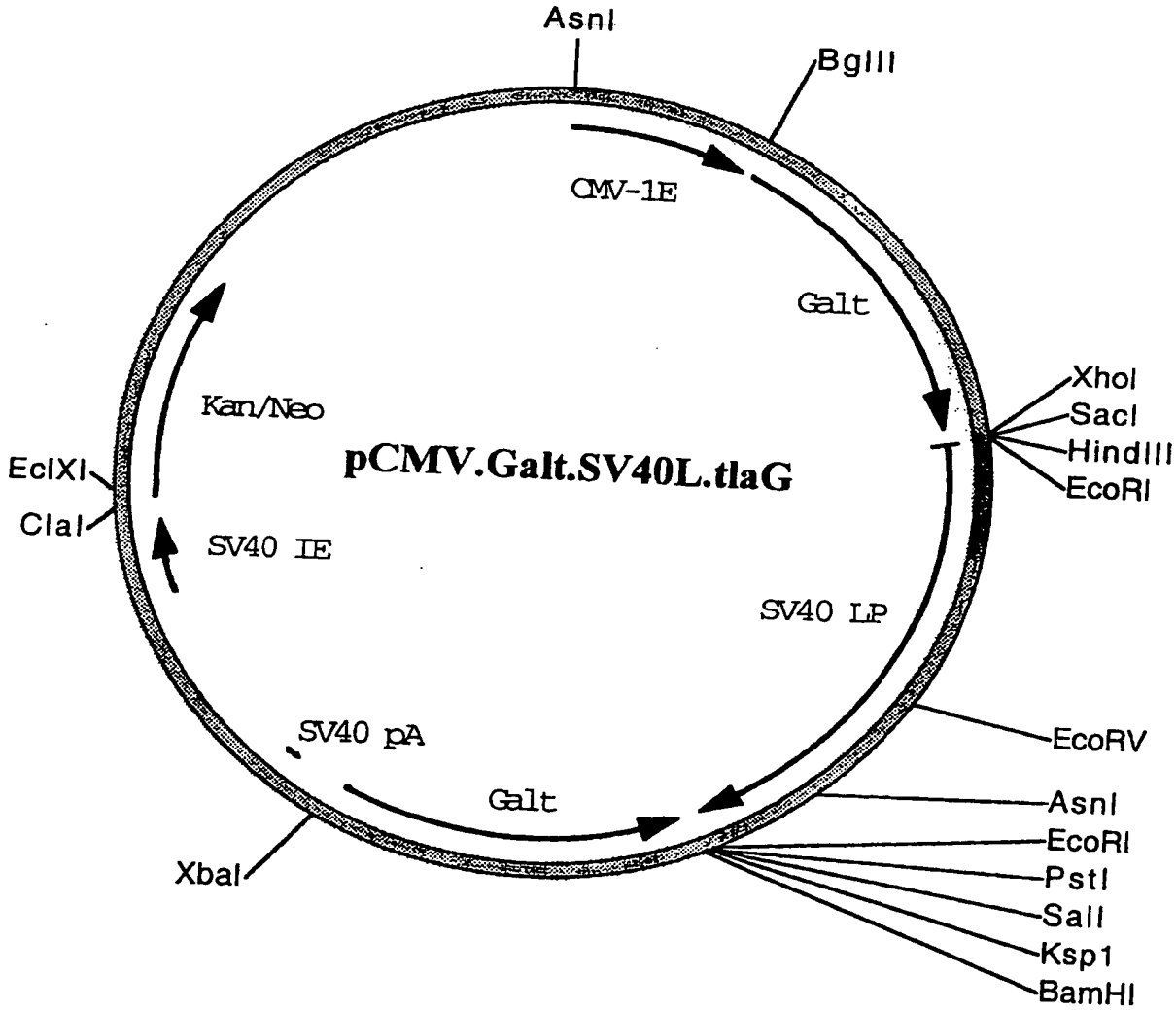

AsnI
BglII
CMV-1E
GalT
XhoI
SacI
HindIII
EcoRI
pCMV.Galt.SV40L.tlaG
Kan/Neo
EclXI
ClaI
SV40 IE
SV40 LP
SV40 pA
EcoRV
GalT
AsnI
EcoRI
PstI
SalI
Ksp1
BamHI
XbaI

FIGURE 38

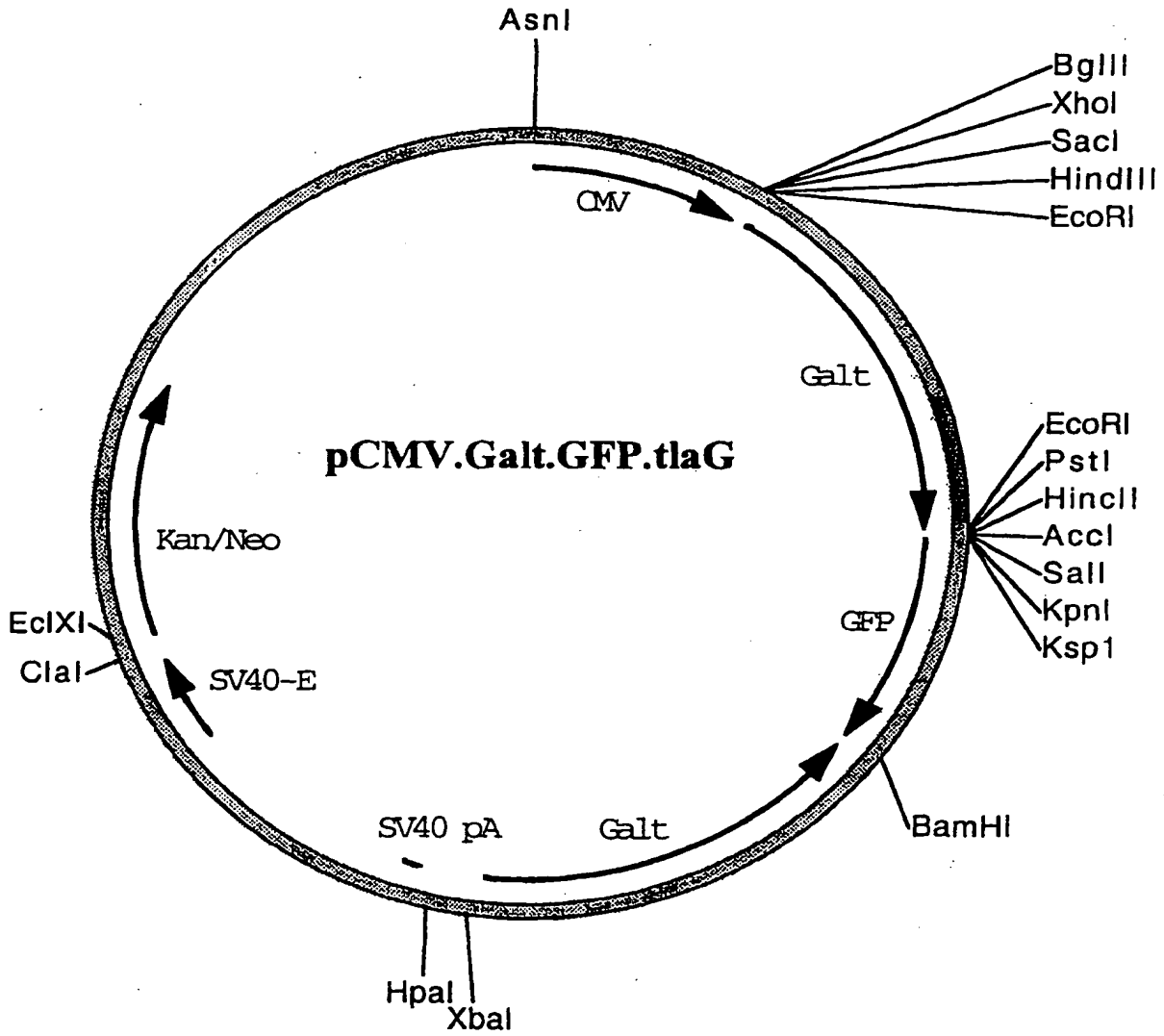

AsnI
BglII
XhoI
SacI
HindIII
EcoRI
CMV
Galt
EcoRI
PstI
HincII
AccI
SalI
KpnI
Ksp1
pCMV.Galt.GFP.tlaG
Kan/Neo
EclXI
ClaI
SV40-E
GFP
SV40 pA
Galt
BamHI
HpaI
XbaI

FIGURE 39

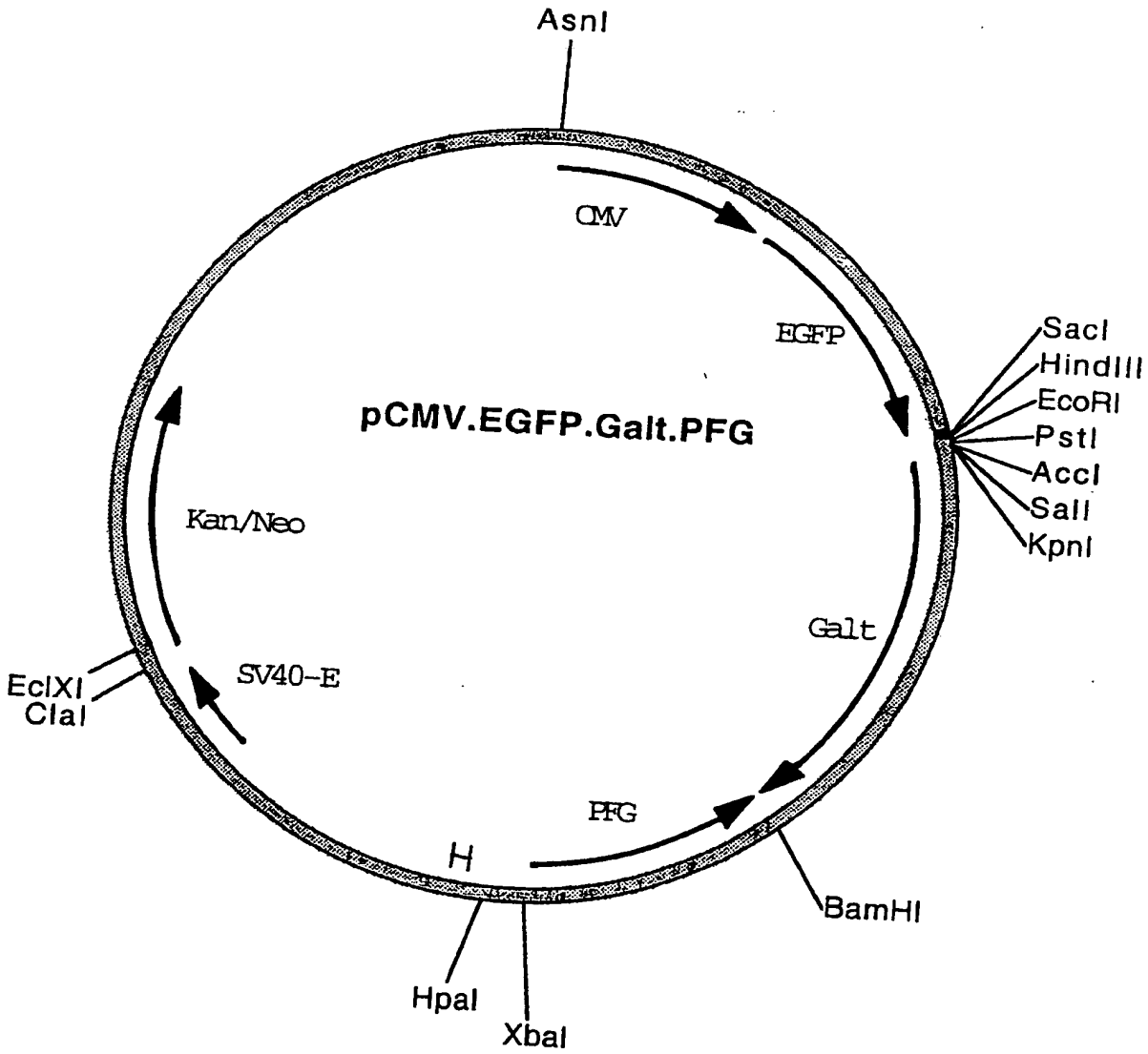

AsnI
CMV
EGFP
SacI
HindIII
EcoRI
PstI
AccI
SalI
KpnI
pCMV.EGFP.Galt.PFG
Kan/Neo
Galt
EclXI
ClaI
SV40-E
PFG
H
BamHI
HpaI
XbaI

FIGURE 40

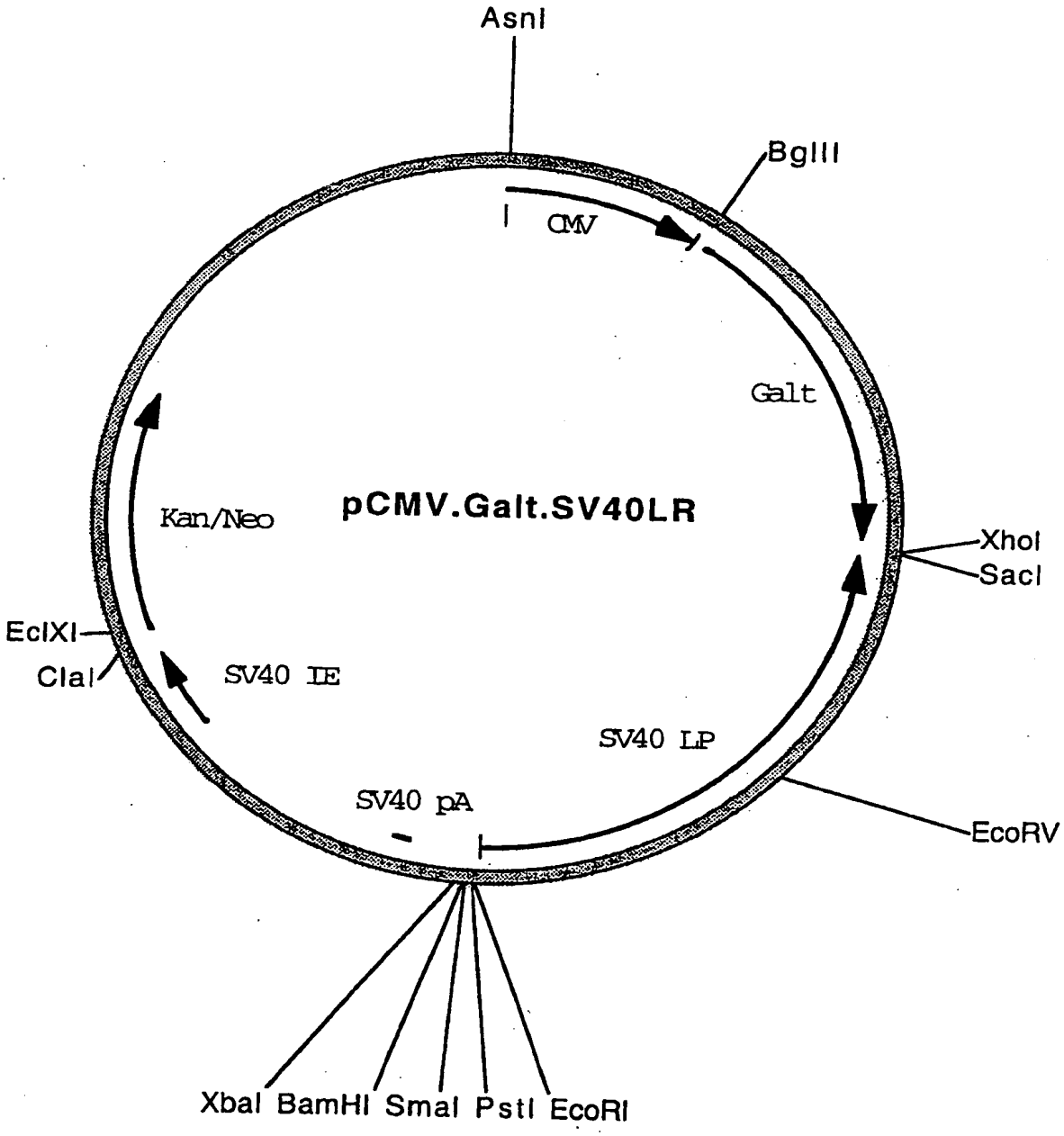

AsnI
BglII
CMV
GalT
pCMV.GalT.SV40LR
Kan/Neo
XhoI
SacI
EclXI
ClaI
SV40 IE
SV40 LP
SV40 pA
EcoRV
XbaI BamHI SmaI PstI EcoRI

FIGURE 41

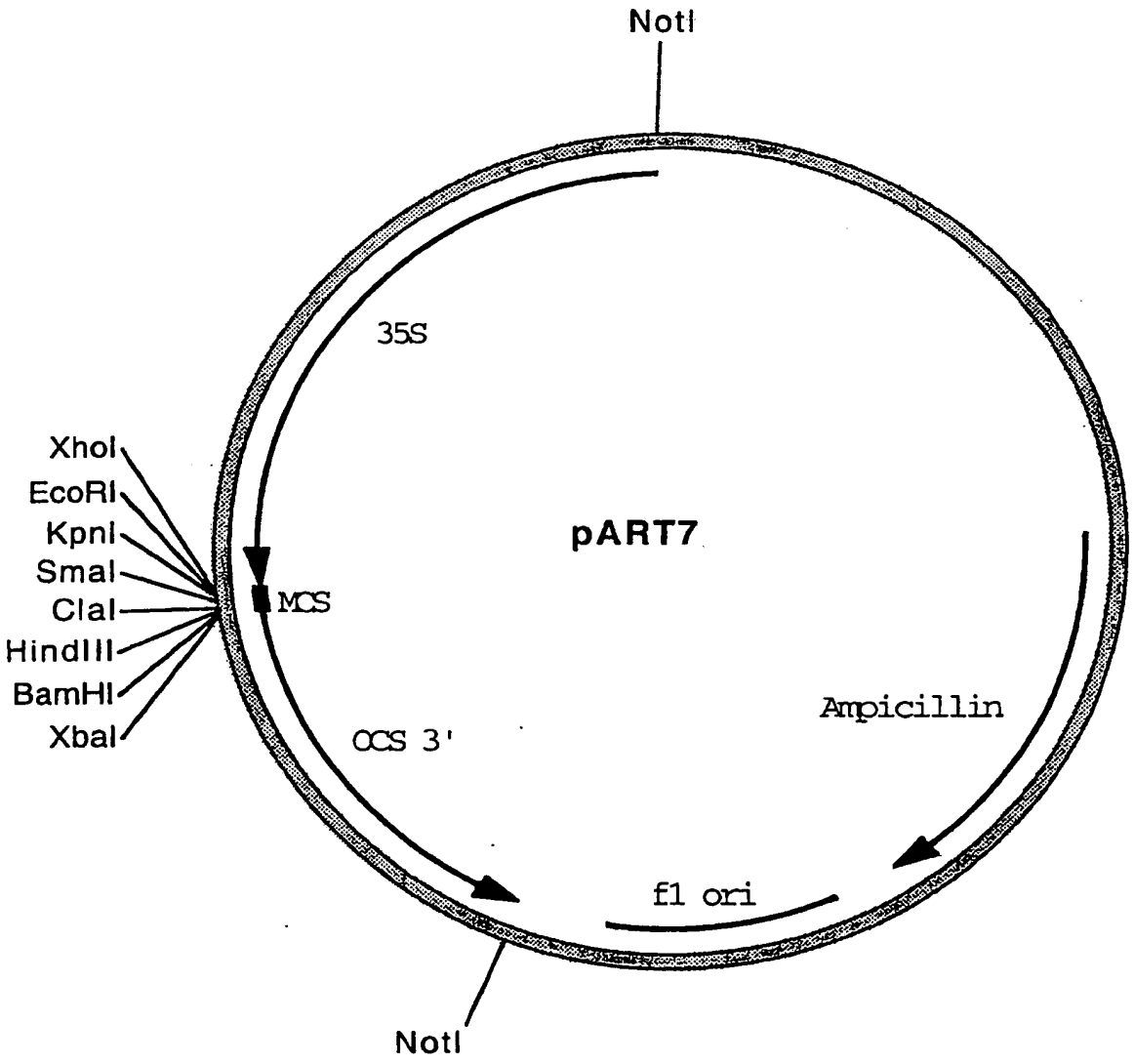

NotI
35S
XhoI
EcoRI
KpnI
SmaI
ClaI
HindIII
BamHI
XbaI
MCS
pART7
OCS 3'
Ampicillin
f1 ori
NotI

FIGURE 42

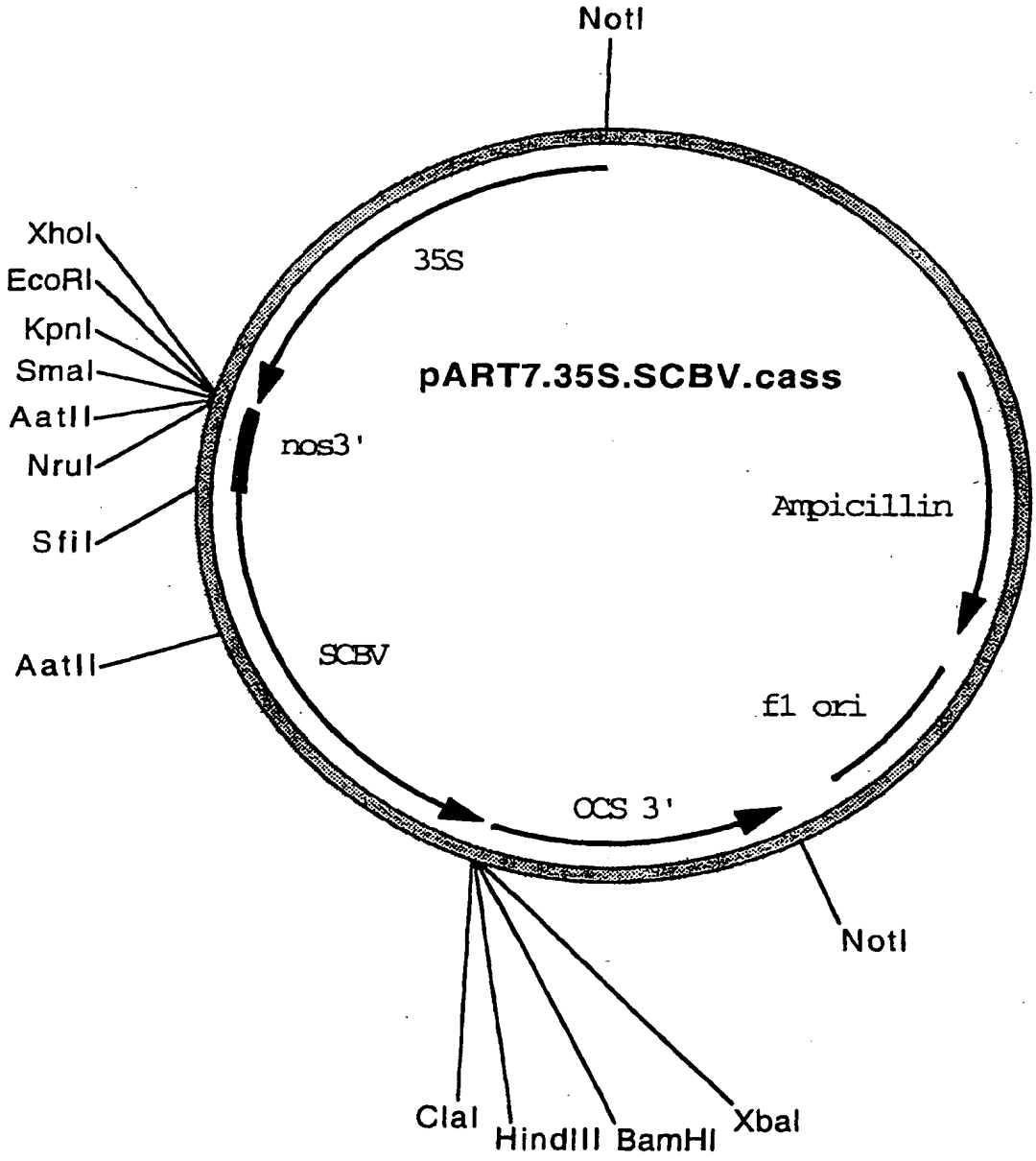
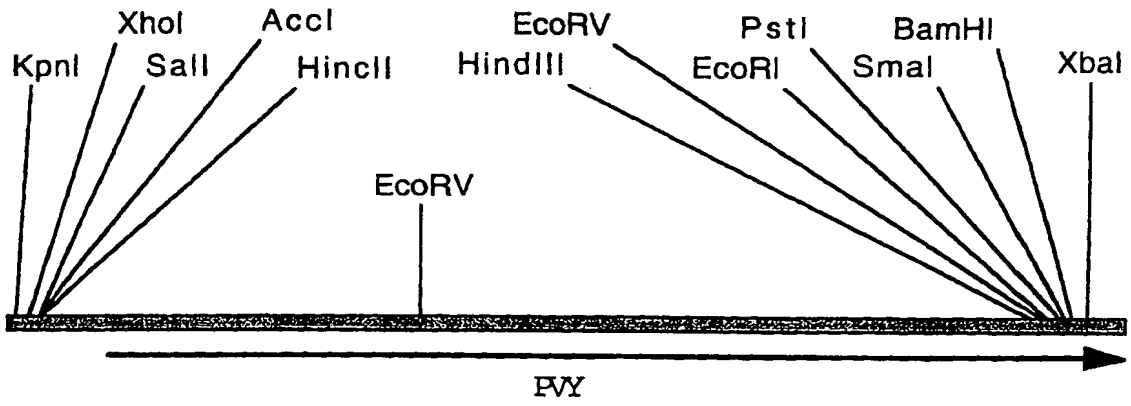

NotI
XhoI
EcoRI
KpnI
SmaI
AatII
NruI
SfiI
AatII
35S
pART7.35S.SCBV.cass
nos3'
Ampicillin
SCBV
f1 ori
OCS 3'
NotI
ClaI
HindIII
BamHI
XbaI

FIGURE 43

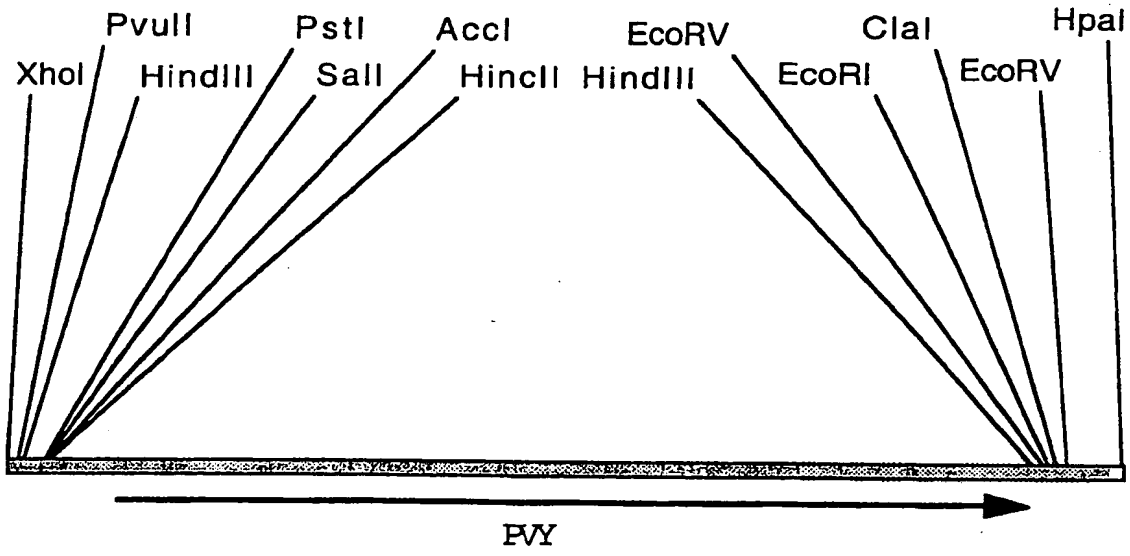
Printed: 22-04-2005 TIPAT 99910592



PBC.PVY

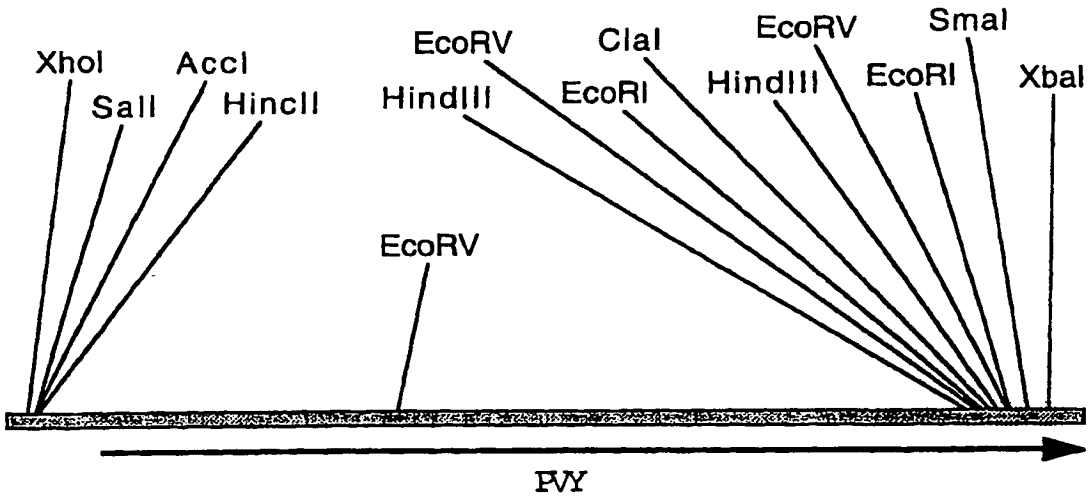
FIGURE 44

20-04-2005



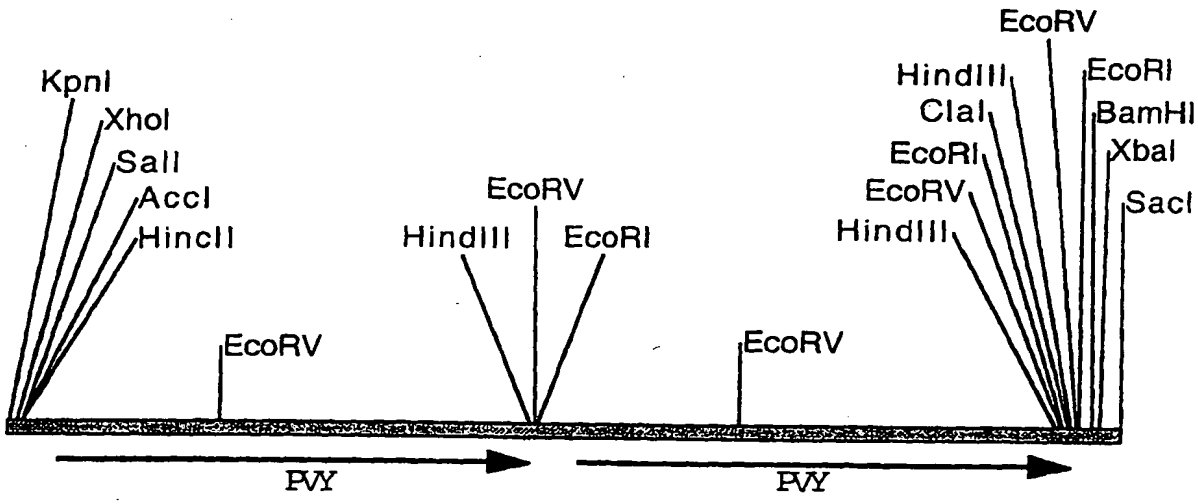
pSP72.PVY

FIGURE 45



ClapBC.PVY

FIGURE 46



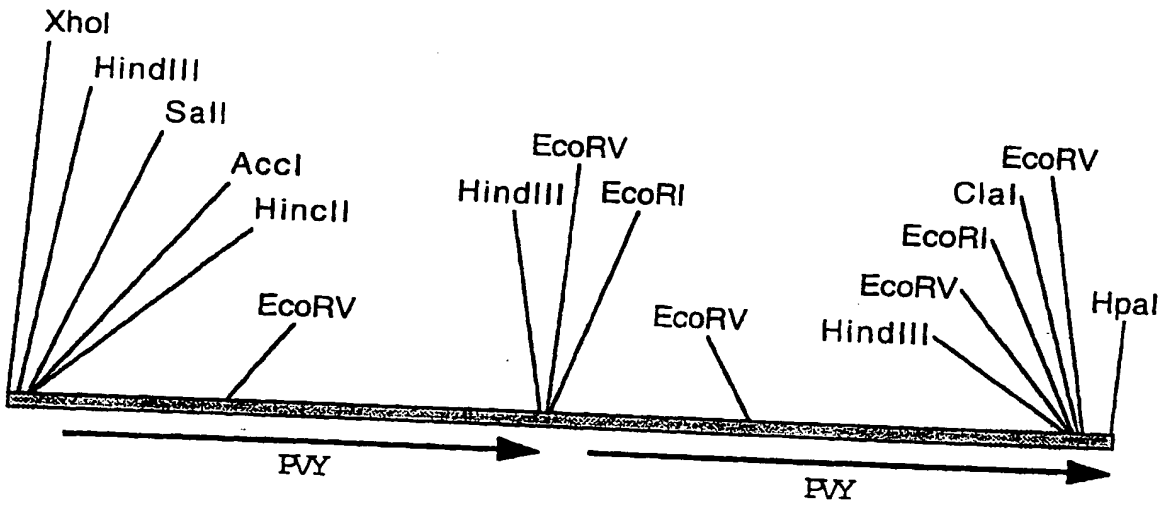
pBC.PVYx2

FIGURE 47

Printed: 22-04-2005

TIPA

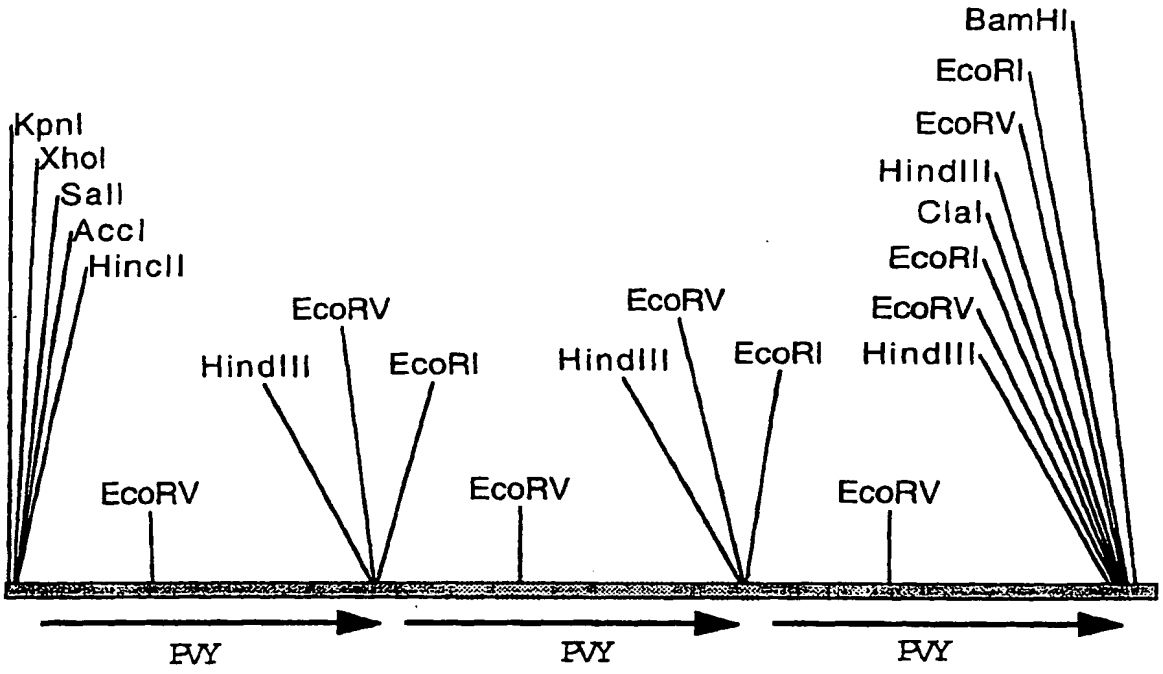
9910592



PSP72.PVYx2

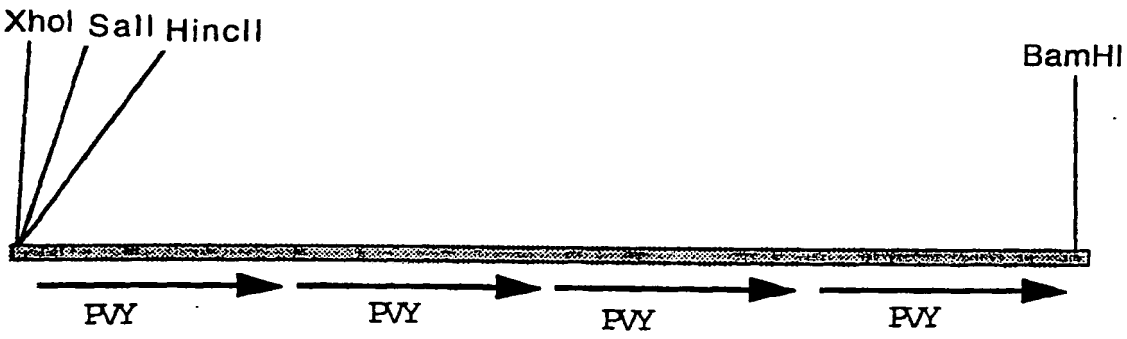
FIGURE 48

20-04-2005



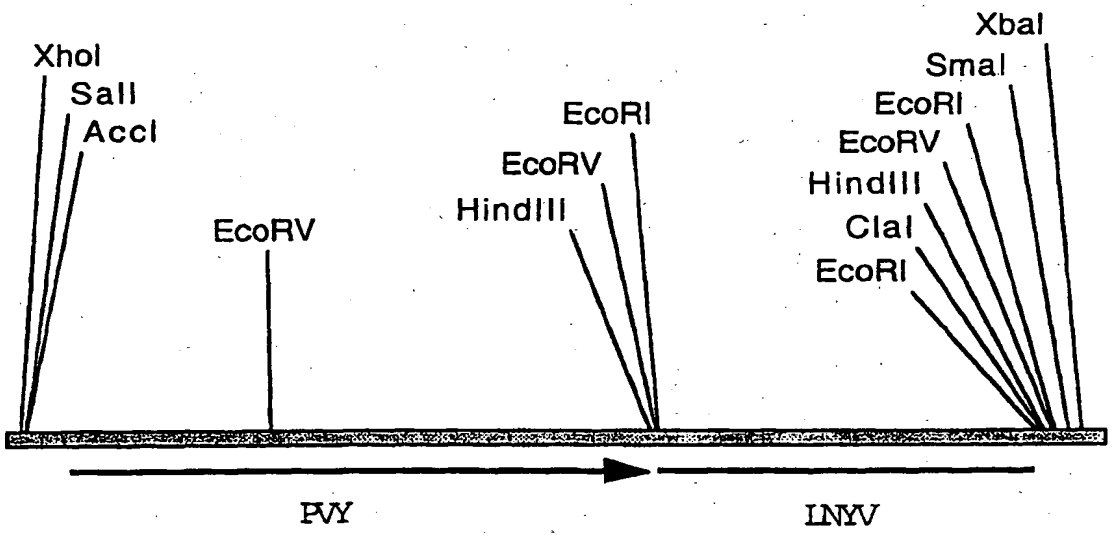
pBC.PVYx3

FIGURE 49



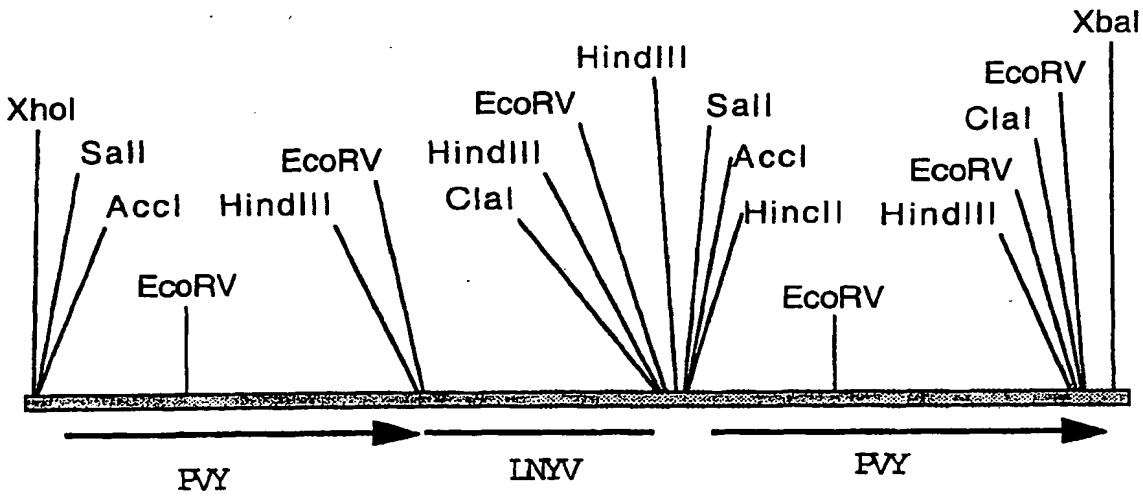
pBC.PVYx4

FIGURE 50



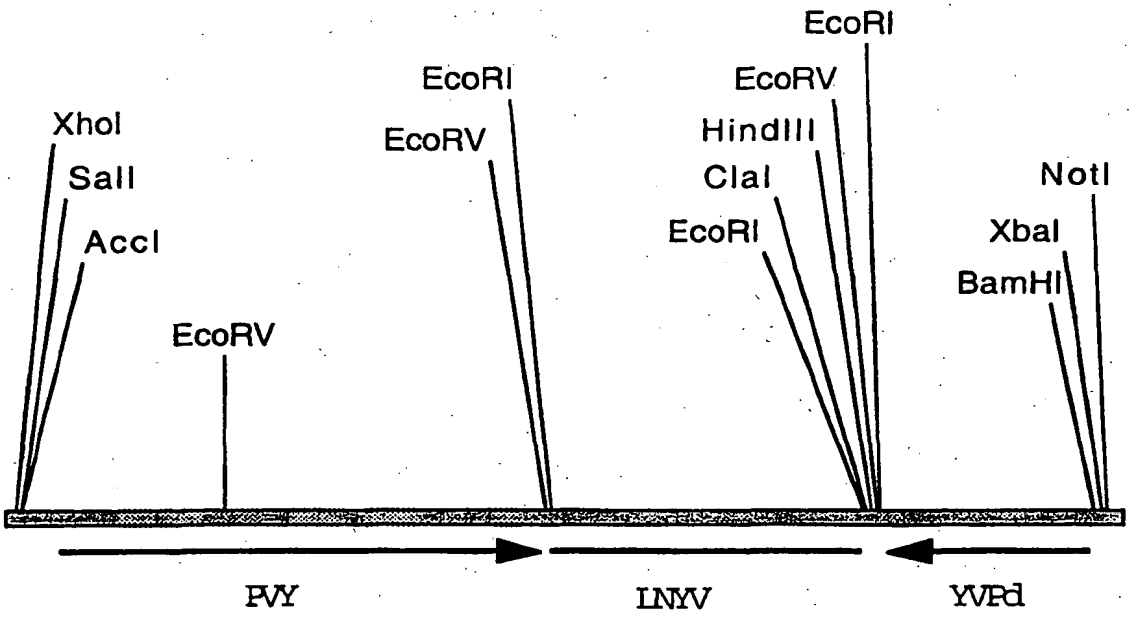
pBC.PVY.LNYV

FIGURE 51



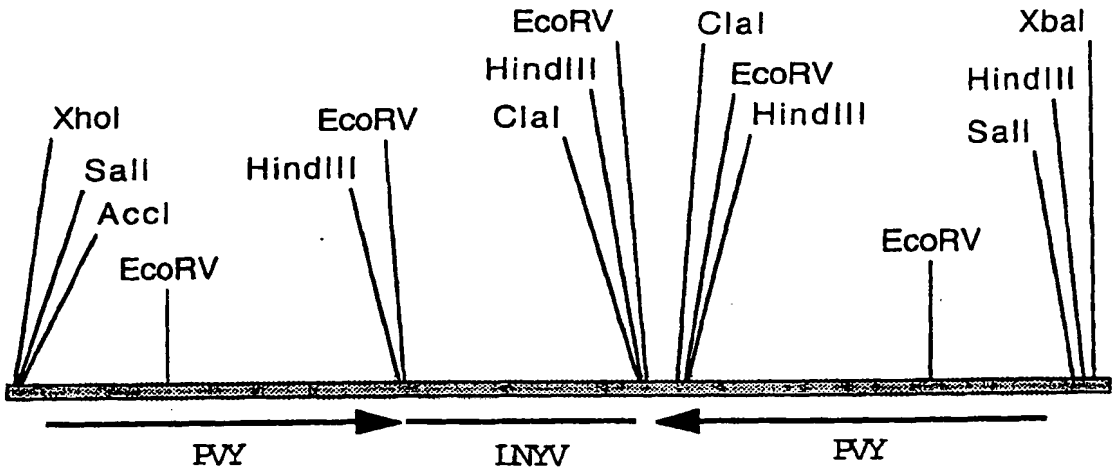
pBC.PVY.LNYV.PYV

FIGURE 52



pBC.PVY.LNYV.YVPΔ

FIGURE 53



pBC.PVY.LNYV.YVP

FIGURE 54

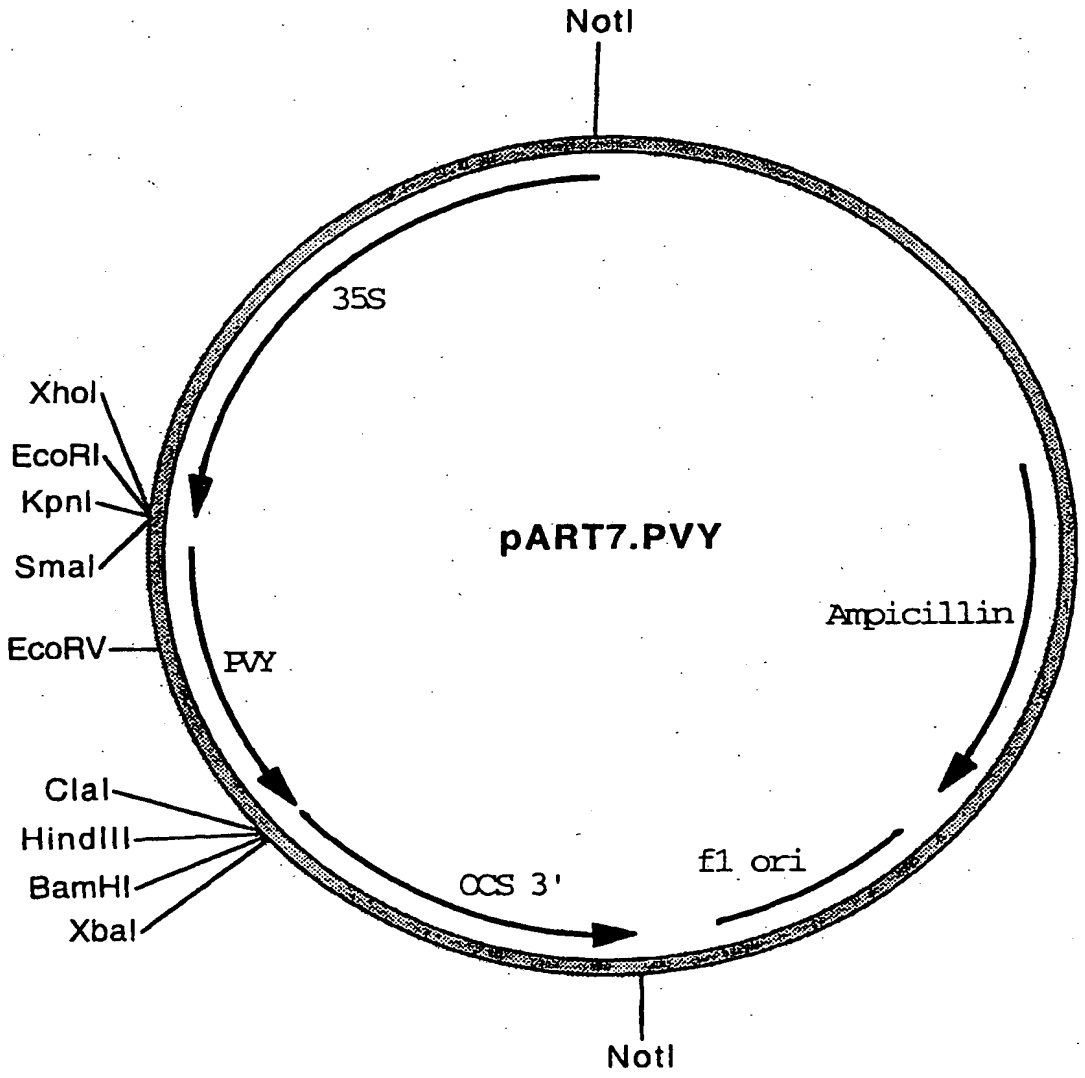

NotI
35S
XhoI
EcoRI
KpnI
SmaI
EcoRV
ClaI
HindIII
BamHI
XbaI
pART7.PVY
PVY
Ampicillin
f1 ori
OCS 3'
NotI

FIGURE 55

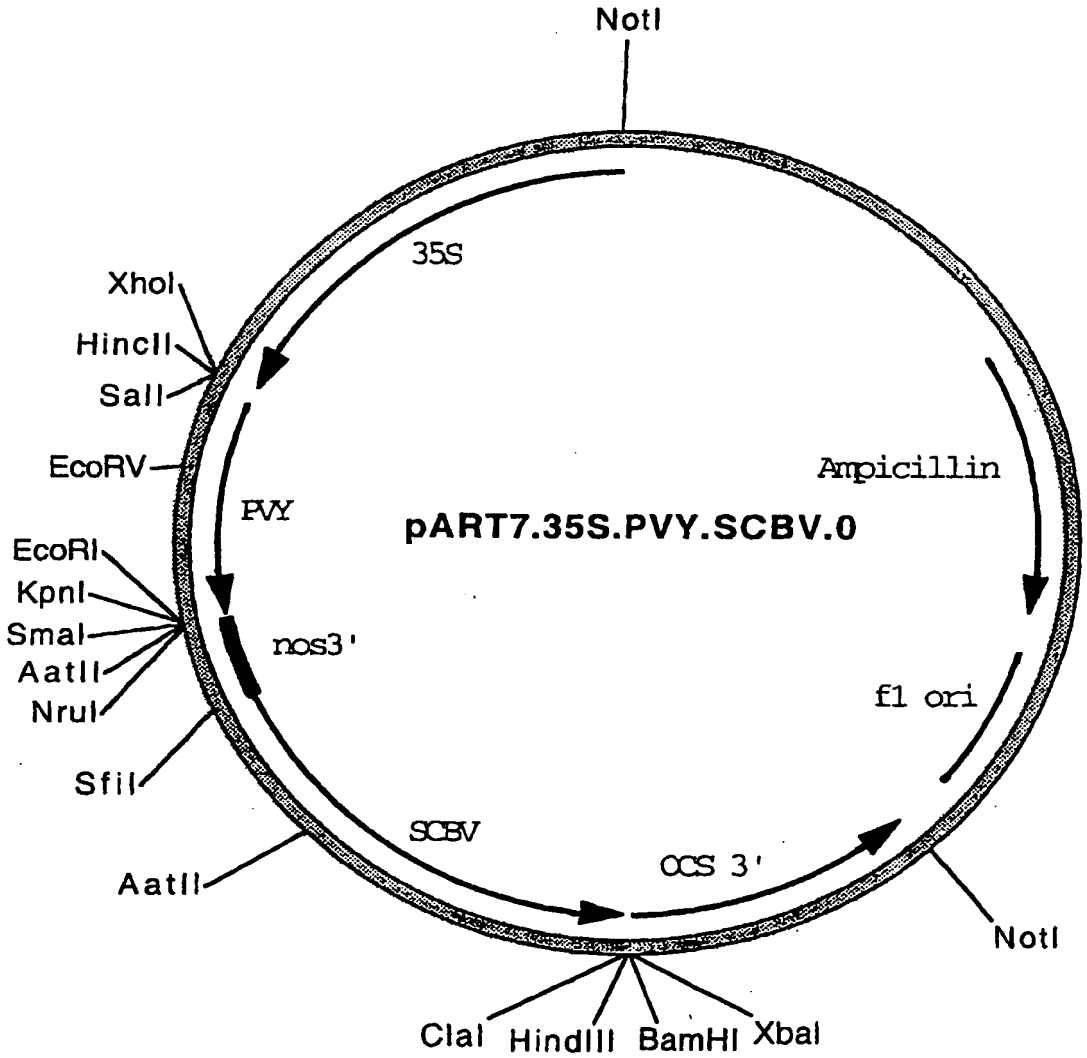

NotI
35S
XhoI
HincII
SalI
EcoRV
PVY
pART7.35S.PVY.SCBV.0
Ampicillin
EcoRI
KpnI
SmaI
AatII
NruI
nos3'
f1 ori
SfiI
SCBV
OCS 3'
AatII
NotI
ClaI
HindIII
BamHI
XbaI

FIGURE 56

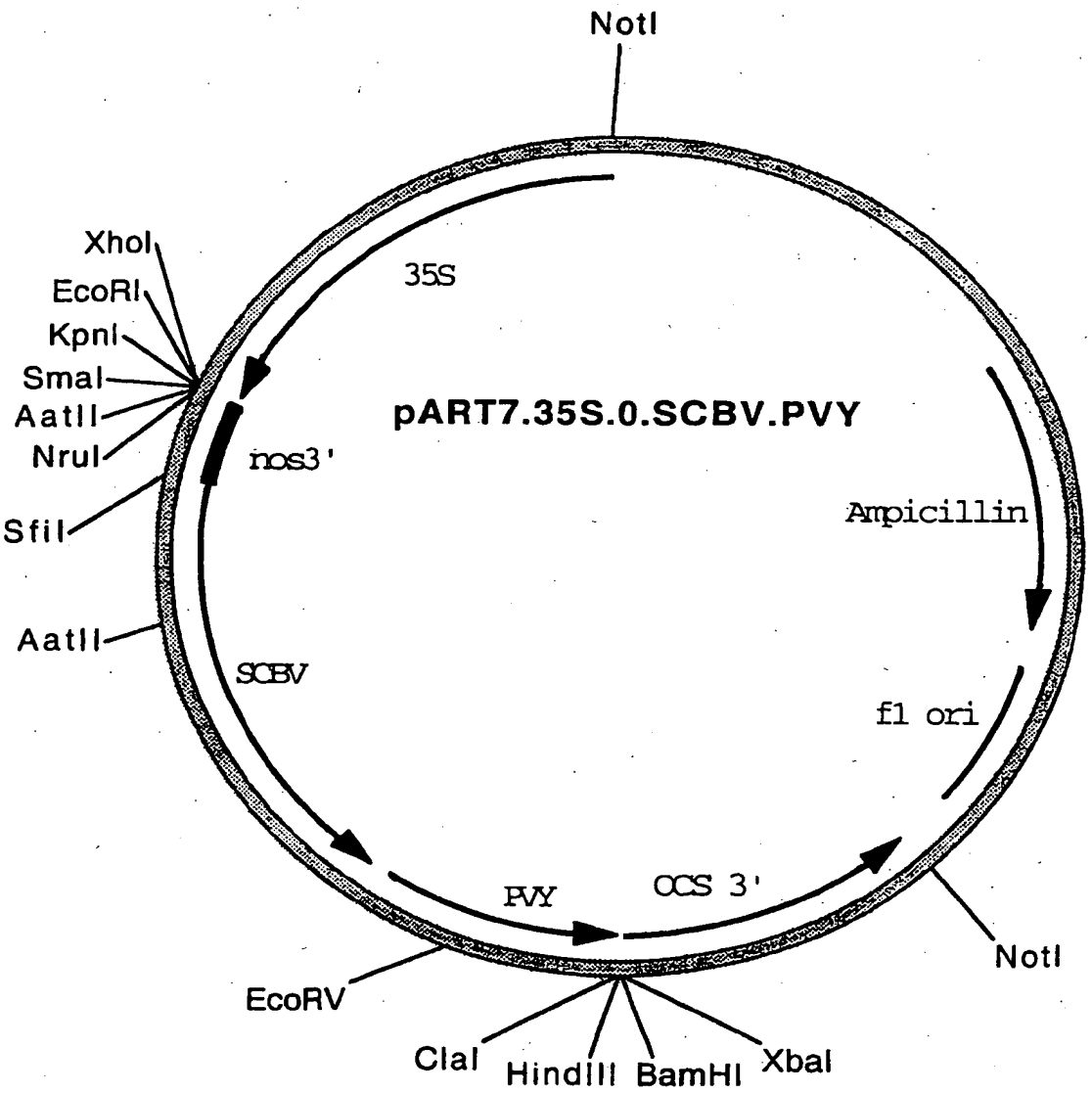

NotI
XhoI
EcoRI
KpnI
SmaI
AatII
NruI
SfiI
AatII
35S
pART7.35S.0.SCBV.PVY
nos3'
Ampicillin
SCBV
f1 ori
PVY
OCS 3'
NotI
EcoRV
ClaI
HindIII
BamHI
XbaI

FIGURE 57

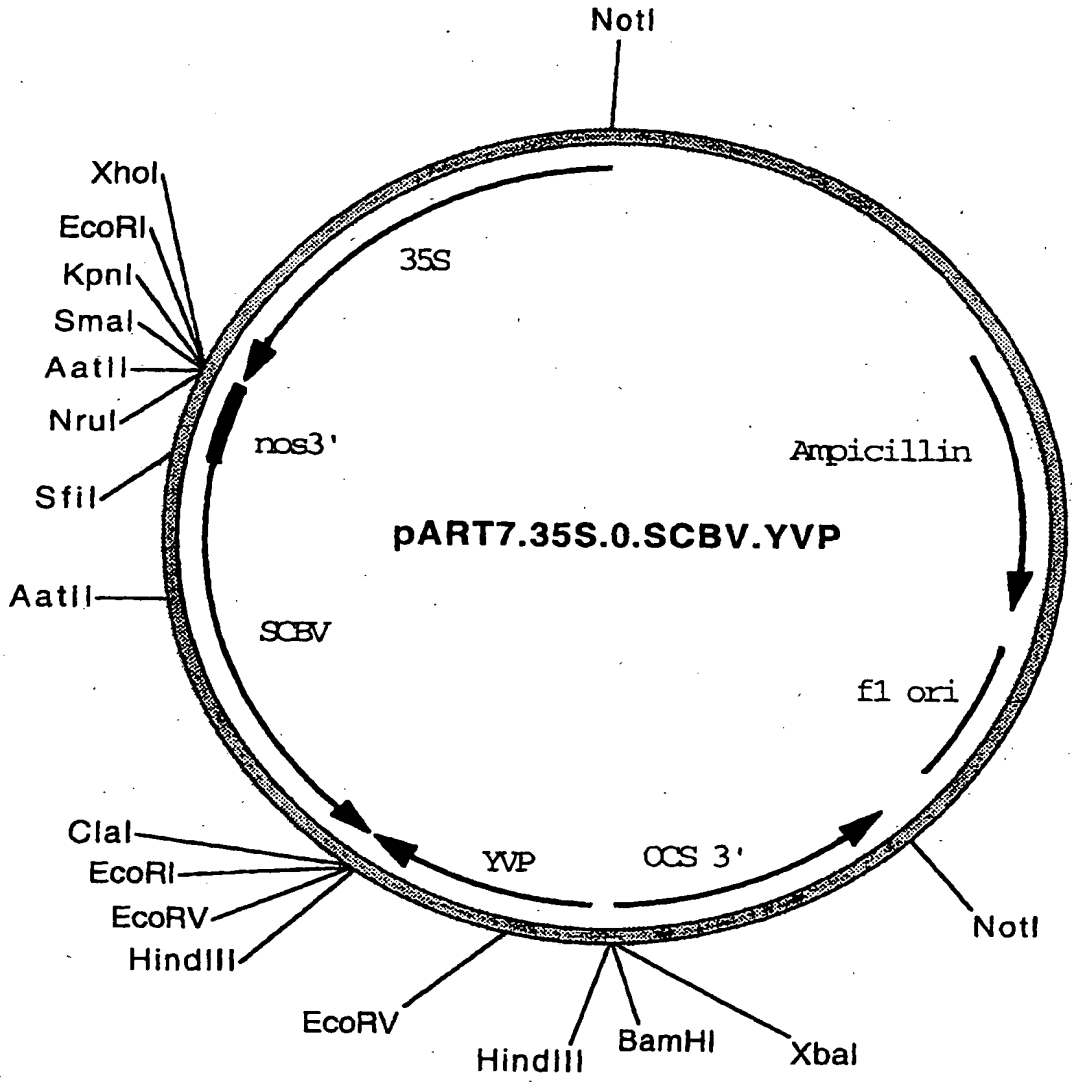

NotI
XhoI
EcoRI
KpnI
SmaI
AatII
NruI
SfiI
AatII
ClaI
EcoRI
EcoRV
HindIII
EcoRV
HindIII
BamHI
XbaI
NotI
35S
nos3'
SCBV
YVP
OCS 3'
Ampicillin
f1 ori
pART7.35S.0.SCBV.YVP

FIGURE 58

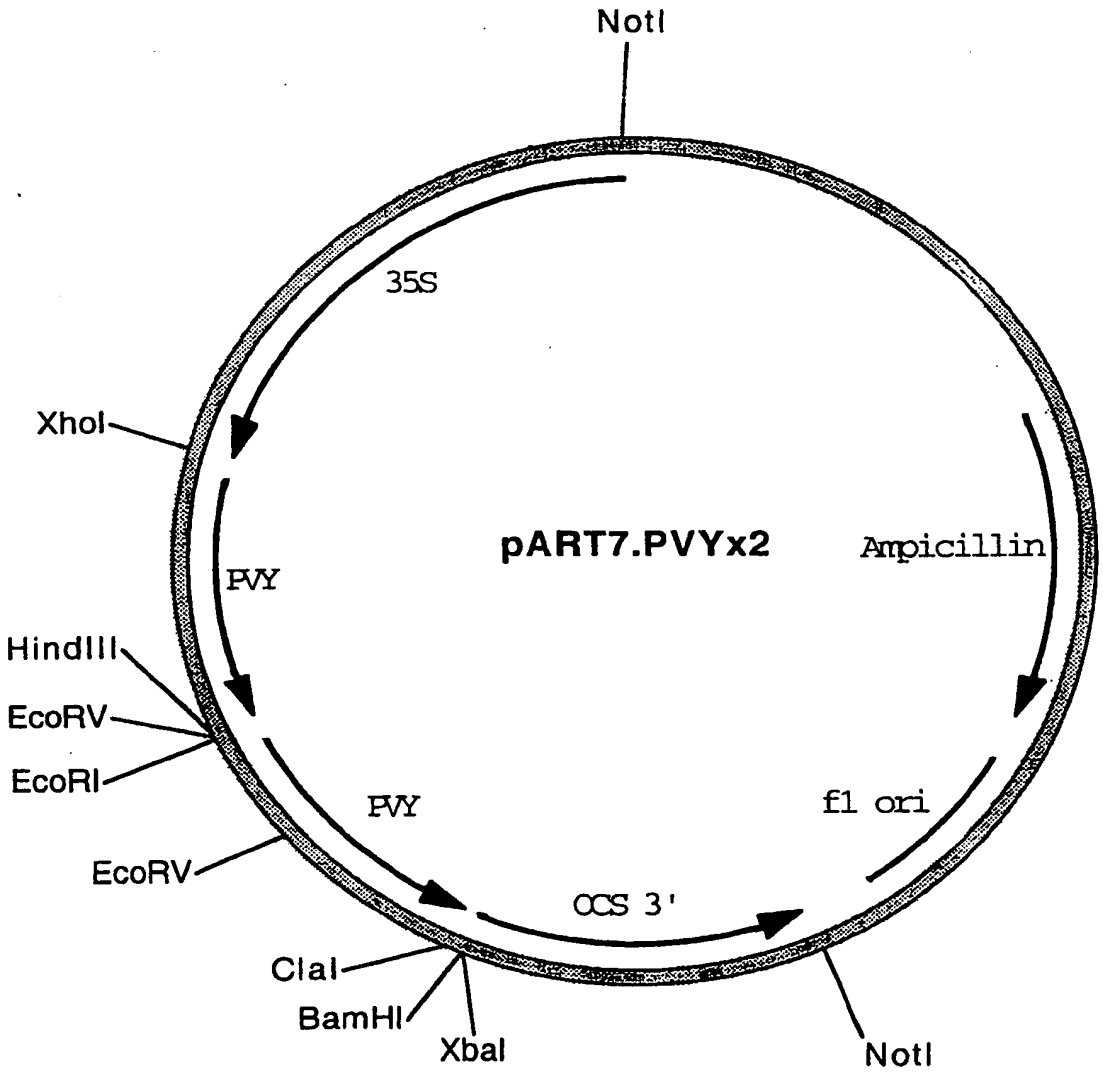

NotI
35S
XhoI
pART7.PVYx2
Ampicillin
PVY
HindIII
EcoRV
EcoRI
PVY
f1 ori
EcoRV
OCS 3'
ClaI
BamHI
XbaI
NotI

FIGURE 59

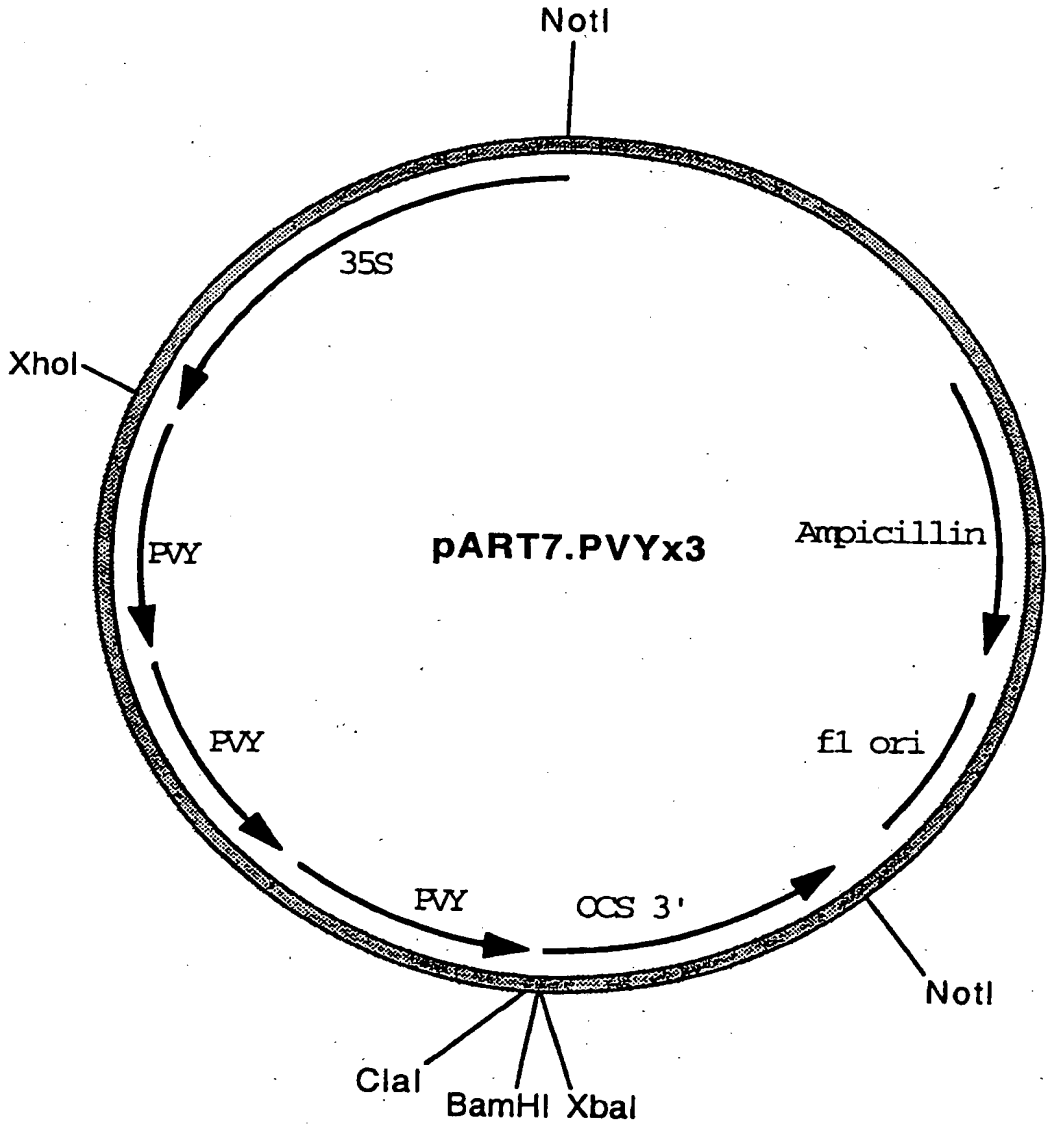

NotI
35S
XhoI
PVY
pART7.PVYx3
Ampicillin
PVY
f1 ori
PVY
OCS 3'
NotI
ClaI
BamHI
XbaI

FIGURE 60

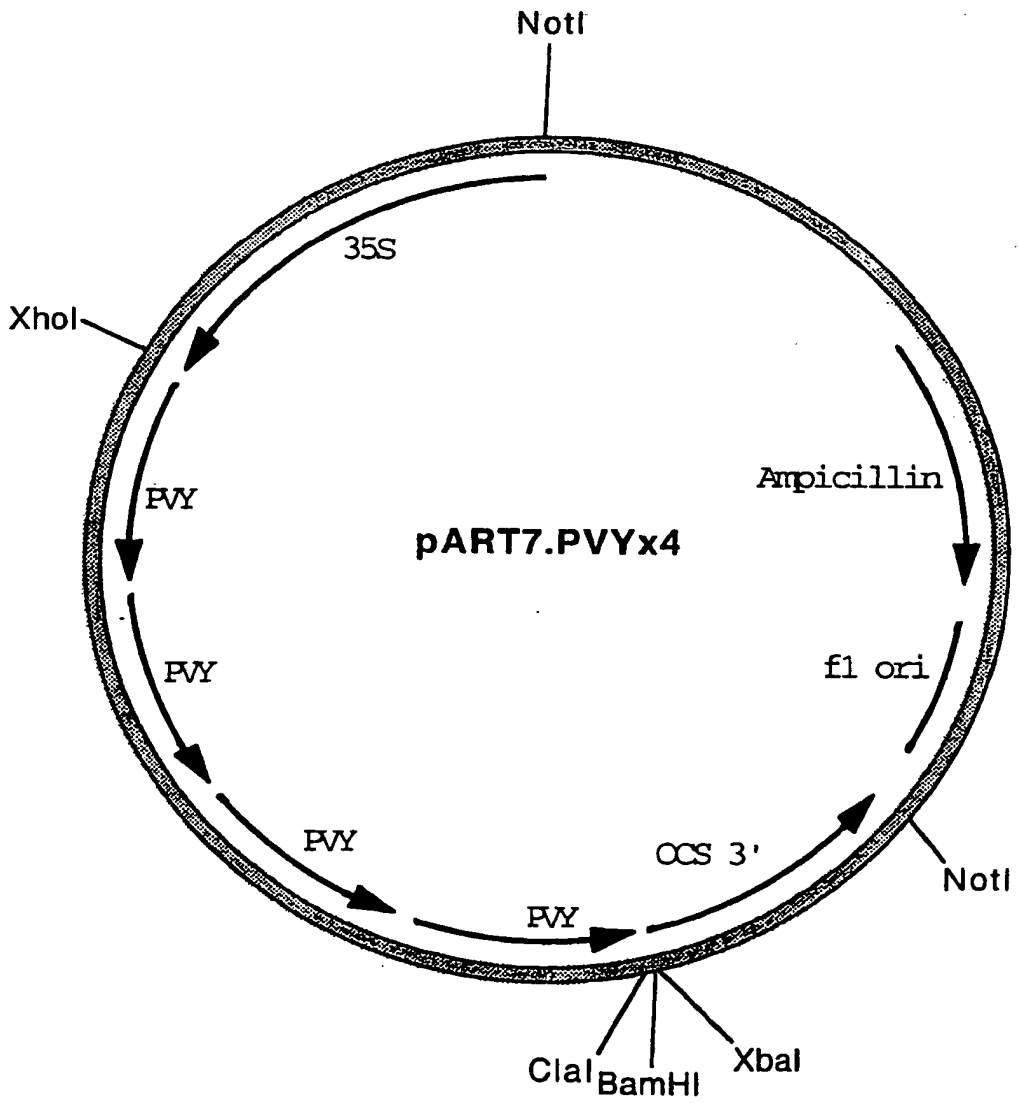

NotI
35S
XhoI
PVY
Ampicillin
pART7.PVYx4
PVY
f1 ori
PVY
OCS 3'
NotI
PVY
ClaI
BamHI
XbaI

FIGURE 61

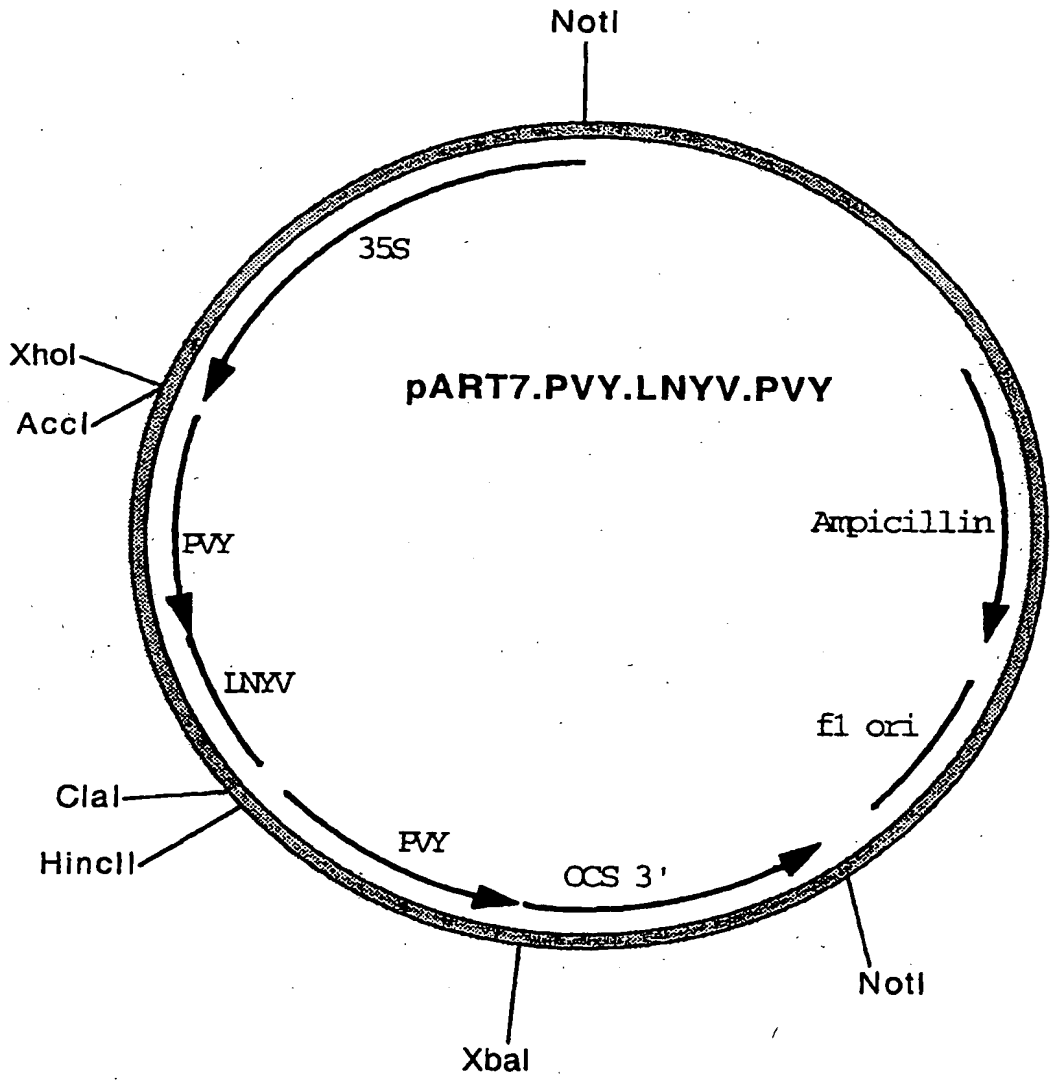

NotI
35S
XhoI
AccI
pART7.PVY.LNYV.PVY
PVY
Ampicillin
LNYV
f1 ori
ClaI
HincII
PVY
OCS 3'
NotI
XbaI

FIGURE 62

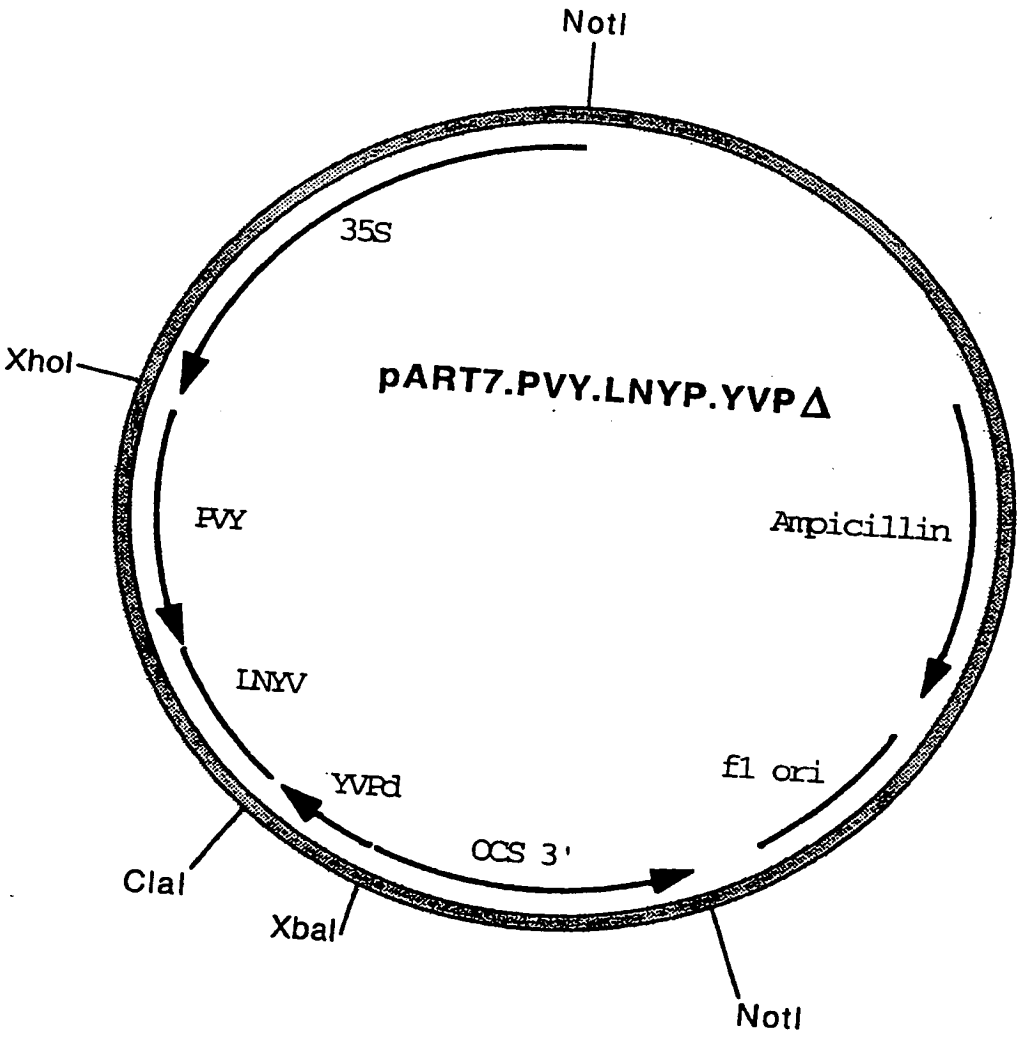

NotI
35S
XhoI
pART7.PVY.LNYP.YVPΔ
PVY
Ampicillin
LNYV
f1 ori
YVPd
OCS 3'
ClaI
XbaI
NotI

FIGURE 63

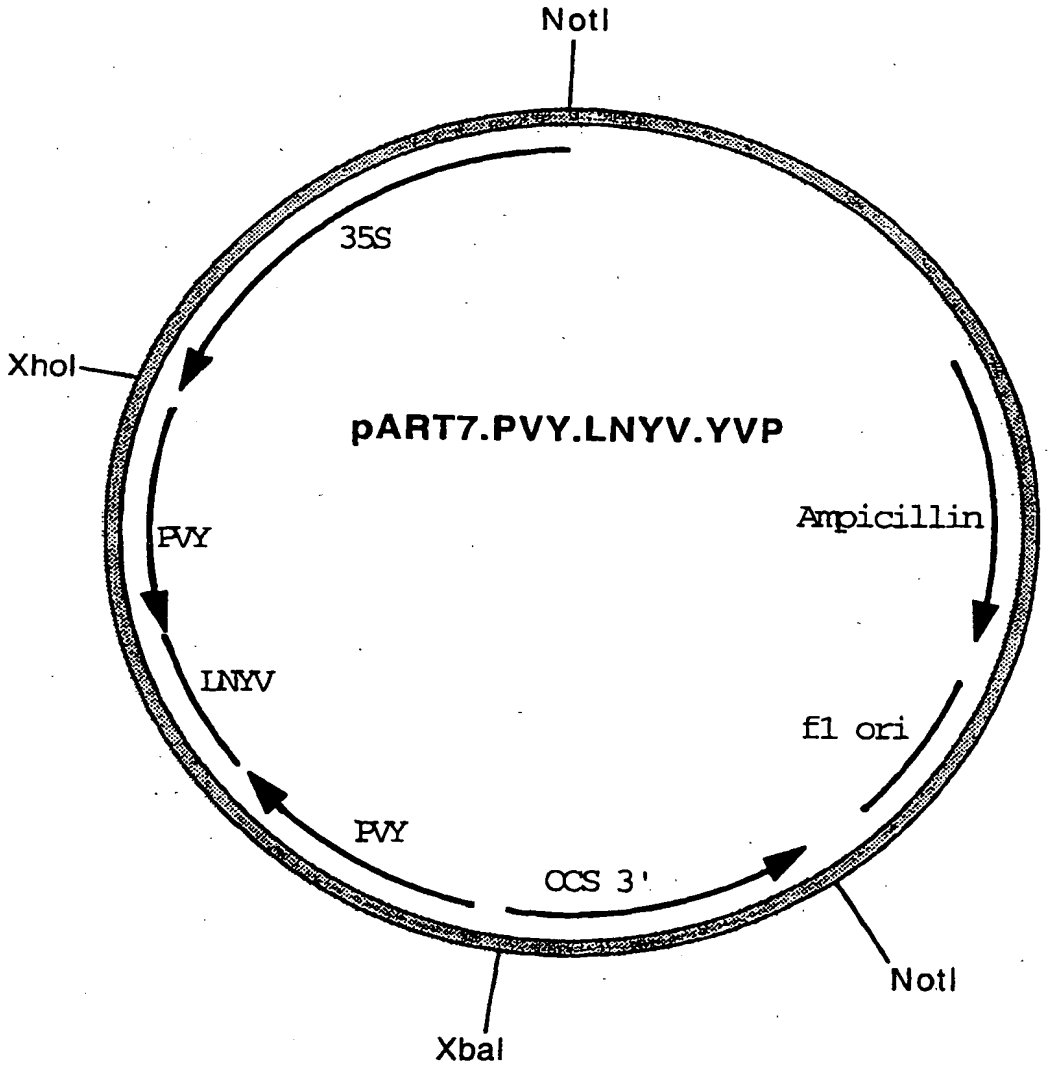

NotI
35S
XhoI
pART7.PVY.LNYV.YVP
PVY
Ampicillin
LNYV
f1 ori
PVY
OCS 3'
NotI
XbaI

FIGURE 64

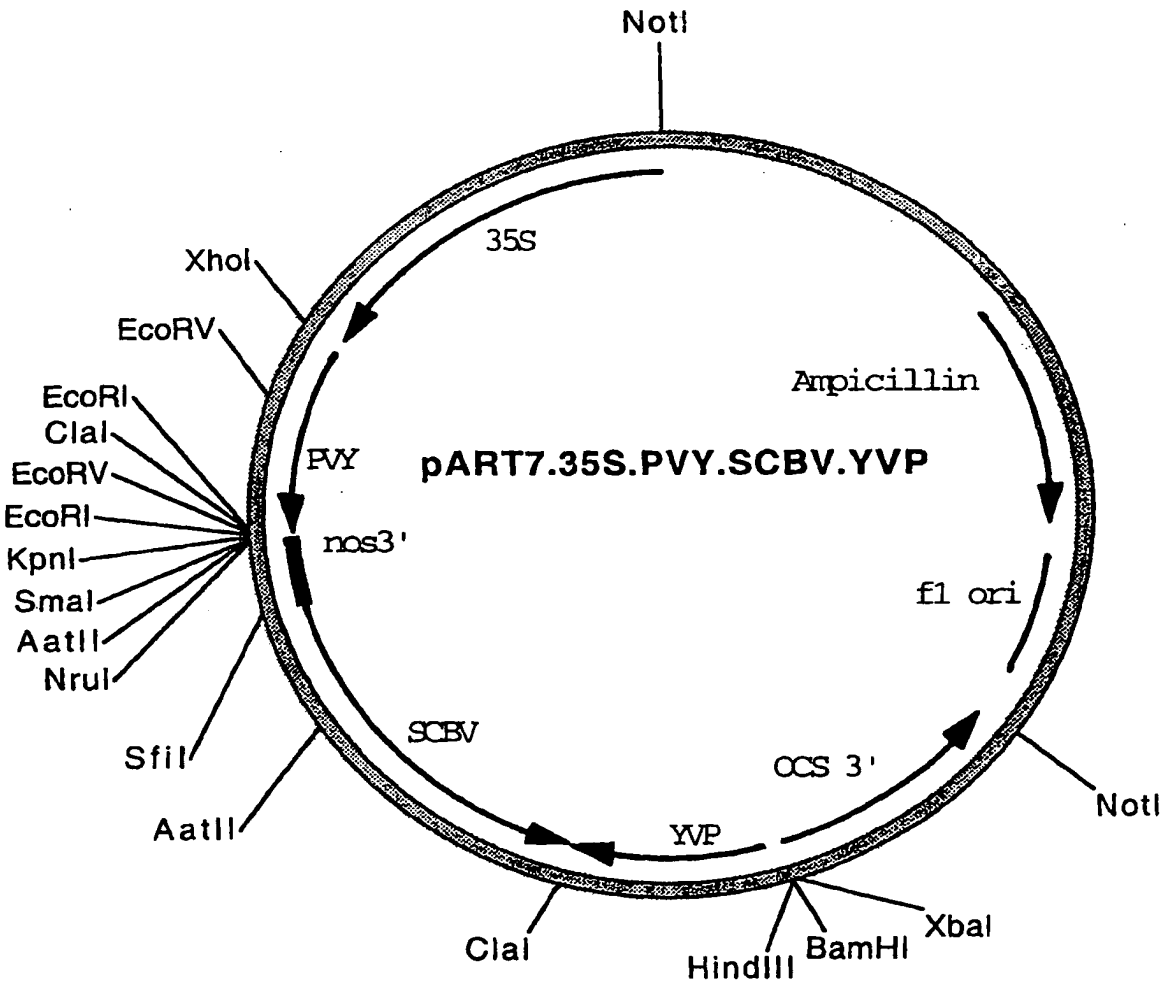

NotI
35S
XhoI
EcoRV
EcoRI
ClaI
EcoRV
EcoRI
KpnI
SmaI
AatII
NruI
SfiI
AatII
PVY
pART7.35S.PVY.SCBV.YVP
nos3'
Ampicillin
f1 ori
SCBV
OCS 3'
NotI
YVP
ClaI
HindIII
BamHI
XbaI

FIGURE 65

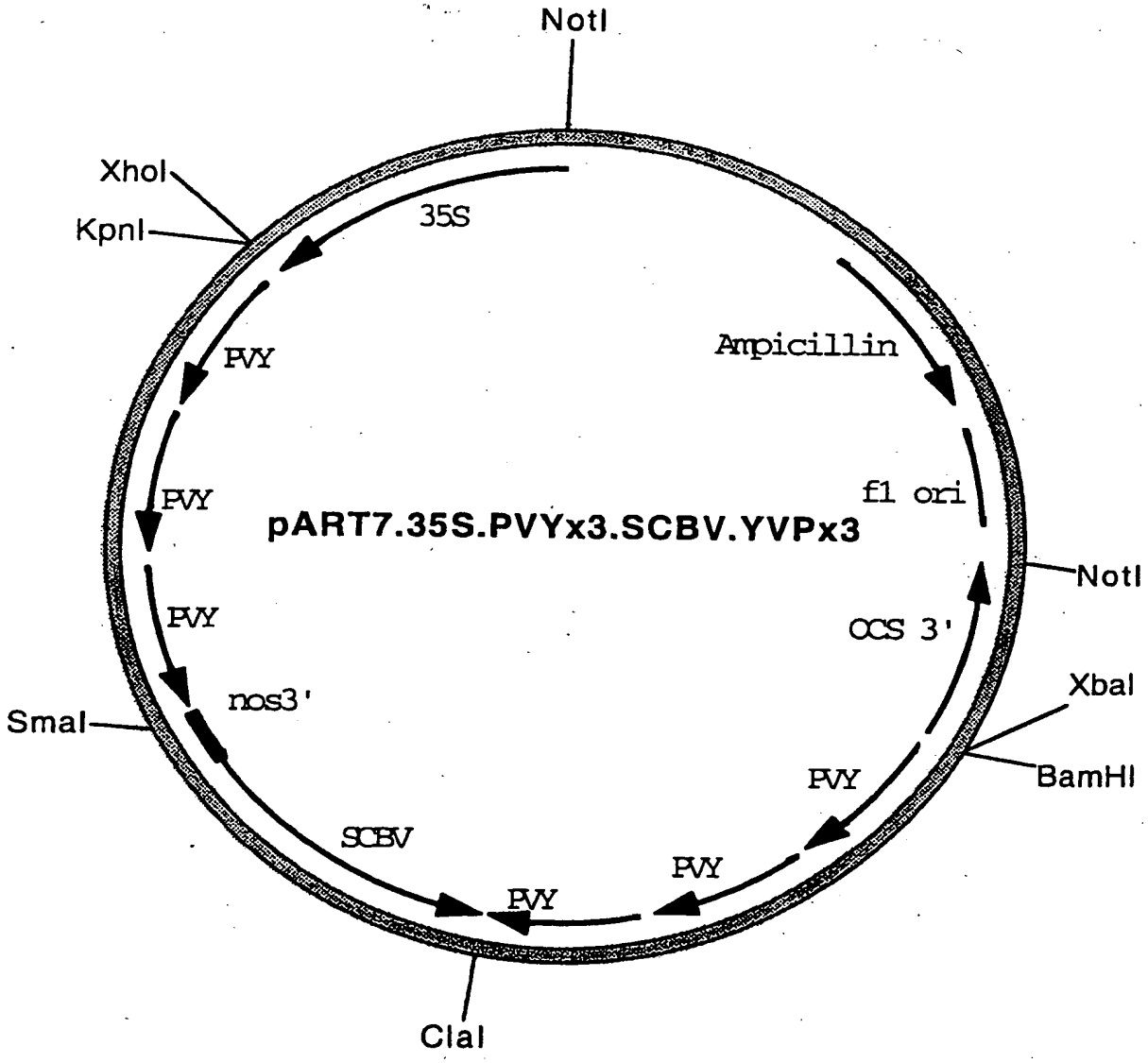

NotI
XhoI
KpnI
35S
PVY
PVY
PVY
pART7.35S.PVYx3.SCBV.YVPx3
Ampicillin
f1 ori
NotI
OCS 3'
XbaI
BamHI
PVY
PVY
PVY
SCBV
nos3'
SmaI
ClaI

FIGURE 66

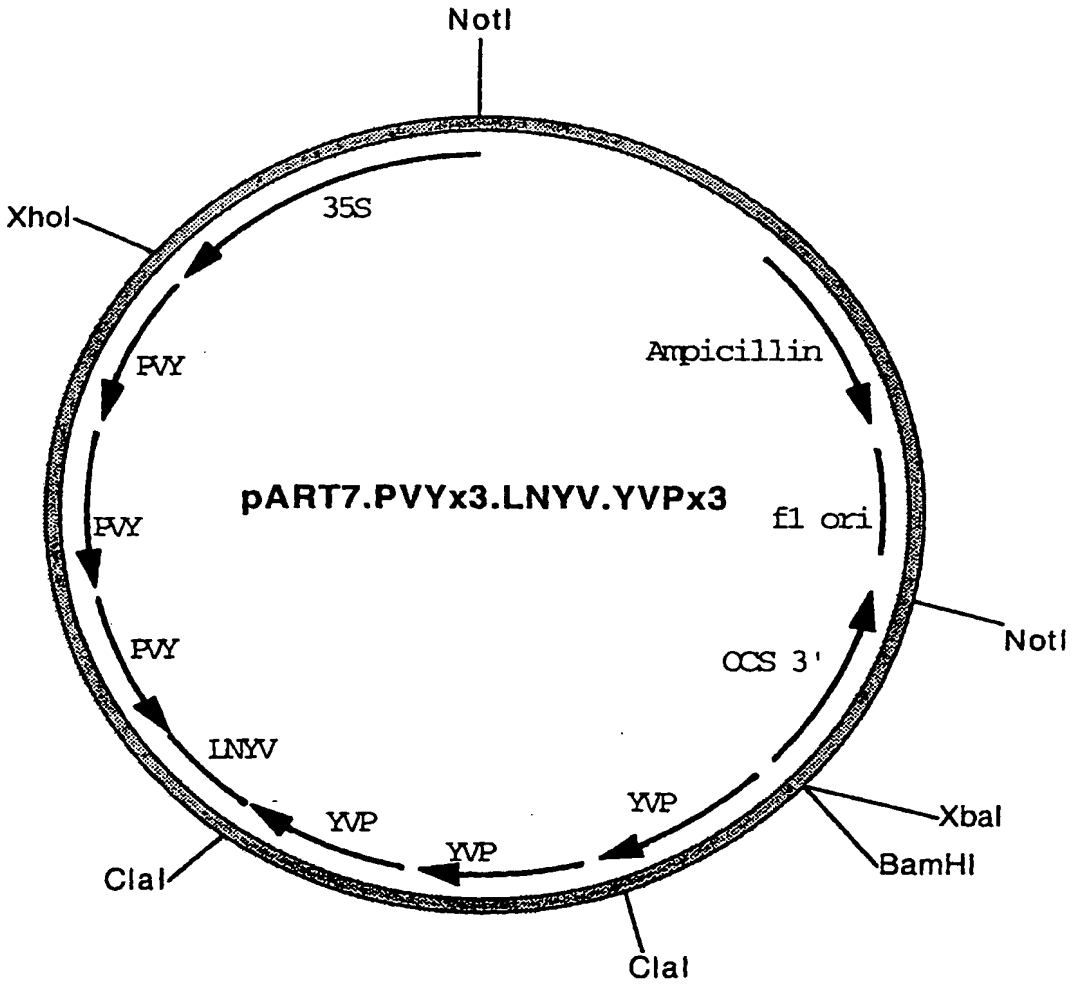

NotI
XhoI
35S
PVY
Ampicillin
pART7.PVYx3.LNYV.YVPx3
PVY
f1 ori
PVY
NotI
OCS 3'
LNYV
YVP
YVP
XbaI
YVP
BamHI
ClaI
ClaI

FIGURE 67

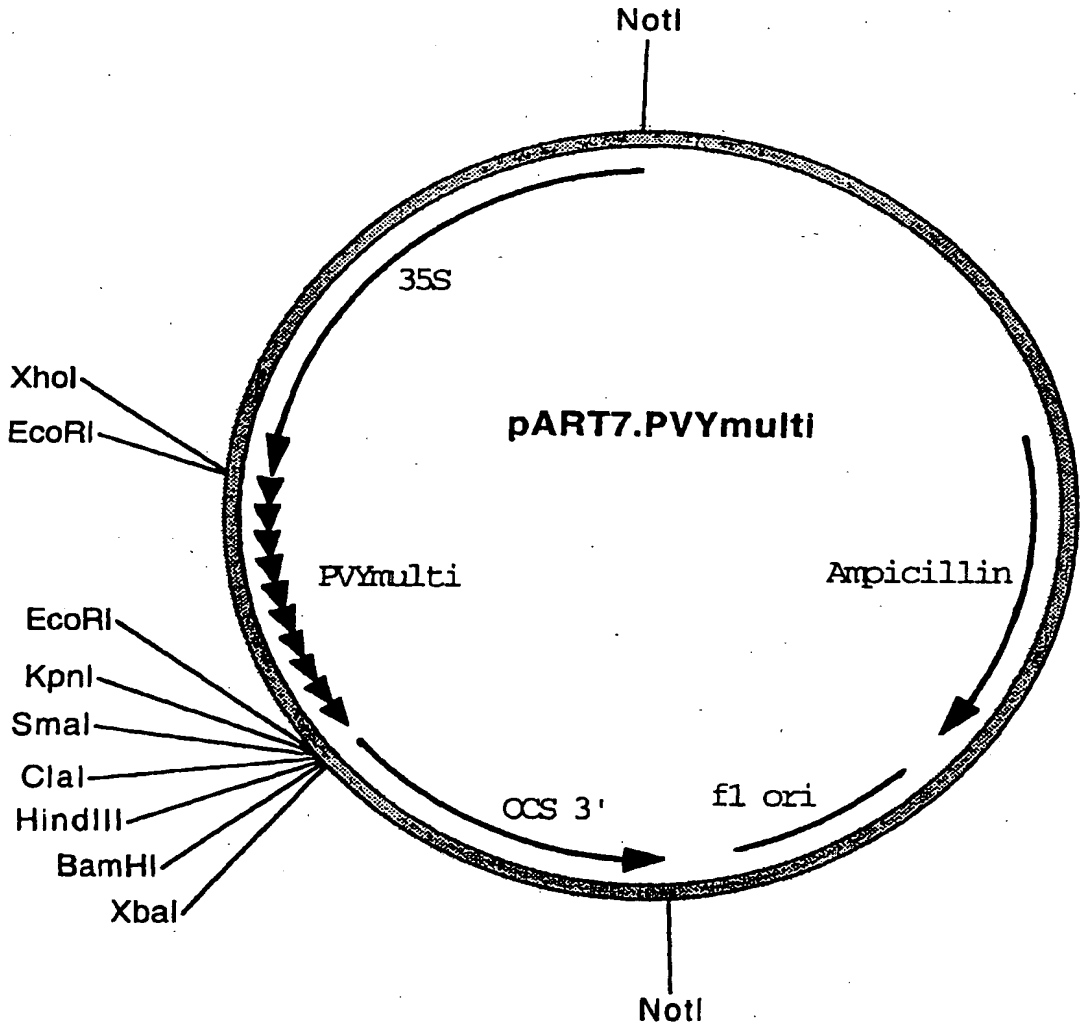

NotI
35S
XhoI
EcoRI
pART7.PVYmulti
PVYmulti
Ampicillin
EcoRI
KpnI
SmaI
ClaI
HindIII
BamHI
XbaI
OCS 3'
f1 ori
NotI

FIGURE 68

IPAU 99910592

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: AgGene Australia Pty.Ltd and The Government of Queensland as represented by Queensland Department of Primary Industries

ii) TITLE OF INVENTION: Synthetic genes and genetic constructs comprising same I

iii) NUMBER OF SEQUENCES: 16

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: DAVIES COLLISON CAVE
(B) STREET: 1 LITTLE COLLINS STREET
(C) CITY: MELBOURNE
(D) STATE: VICTORIA
(E) COUNTRY: AUSTRALIA
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:AU provisional
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: HUGHES EL, JOHN E L

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +61 3 9254 2777
(B) TELEFAX: +61 3 9254 2770
(C) TELEX: AA 31787

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGCAGATCT AACAATGGCA GGACAAATCG AGTACATC 38

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCGGGATCC TCGAAAGAAT CGTACCACTT C 31

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGCGGATCC TTAGAAAGAA TCGTACCAC 29

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGCAGATCT GGACAAATCG AGTACATC 28

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGATCTGTAA ACGGCCACAA GTTCAG 26

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

Printed 22-04-2005 TIPAT 99910592

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGATCCTTGT ACAGCTCGTC CATGCC 26

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTCGACAATA AAATATCTTT ATTTTCATTA CATCTGTGTG TTGGTTTTTT GTGTGATTTT 60

TGCAAAAGCC TAGG 74

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCGACGTTT AGAGCAGAAG TAACACTTCC G 31

30 20-04-2005

Printed: 22-04-2005 TIPA 1 99910592

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCGGGGCTT AGTGTAAAAC AGGCTGAGAG 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCCGGGCAAA TCCCAGTCAT TTCTTAGAAA C 31

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

20-04-2005

Printed: 22-04-2005 TIPAT 99910592

CGGCAGATCT AACAATGGCA GGACAAATCG AGTACATC 38

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCCGGGATCC TCGAAAGAAT CGTACCACTT C 31

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGGCGGATCC TTAGAAAGAA TCGTACCAC 29

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20-04-2005

Printed: 22-04-2005 TIPAT 99910592

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGGCAGATCT GGACAAATCG AGTACATC 28

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCCGGGGCTT AGTGTAAAAC AGGCTGAGAG 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCCGGGCAAA TCCCAGTCAT TTCTTAGAAA C 31

20-04-2005

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00195

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (WPAT - WORLD PATENTS INDEX) AND CHEMICAL ABSTRACTS. KEYWORDS BELOW.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
MEDLINE. KEYWORDS BELOW.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, Medline, Chemical Abstracts. Keywords: homology, transcriptional, posttranscriptional, RNA mediated, epigenetic, silencing, cosuppression, virus resistance

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Cell, 96(3), 5 February 1999, Grant, "Dissecting the Mechanisms of Posttranscriptional Gene Silencing: Divide and Conquer", pages 303-6	1-49,57
X Y	Developmental Genetics, 22(1), 1998, Que et al., "Homology-Based Control of Gene Expression Patterns in Petunia Flowers", pages 100-9	1-16,23,24,35,36,47-49,57 17-22,25-34,37-49,57
X Y	Plant Molecular Biology, 22(6), 1993, Assaad et al., "Epigenetic repeat-induced gene silencing (RIGS) in *Arabidopsis*", pages 1067-85	1-16,23,24,35,36,47-49,57 17-22,25-34,37-49,57

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 April 1999	10 MAY 1999
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer CHRISTOPHER LUTON Telephone No.: (02) 6283 2256

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00195

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	The Plant Journal, 15(6), 1998, Hamilton et al., "A transgene with repeated DNA causes high frequency, post-transcriptional suppression of ACC-oxidase gene expression in tomato", pages 737-46	1-49,57
X	Annals of Botany, 79(1), 1997, Stam et al., "The Silence of Genes in Transgenic Plants", pages 3-12	1-49,57
X Y	Genetics, 147(3), 1997, Dorer and Henikoff, "Transgene Repeat Arrays Interact With Distant Heterochromatin and Cause Silencing in *cis* and *trans*", pages 1181-90	1-16,23,24,35,36,47-49,57 17-22,25-34,37-49,57
X Y	Cell, 77(7), 1994, Dorer and Henikoff, "Expansions of Transgene Repeats Cause Heterochromatin Formation and Gene Silencing in Drosophila, pages 993-1002	1-16,23,24,35,36,47-49,57 17-22,25-34,37-49,57
X	Plant Cell, Volume 8, 1996, Sijen et al., "RNA-Mediated Virus Resistance: Role of Repeated Transgenes and Delineation of Target Regions", pages 2277-94	1-49,57
P,X P,Y	WO, 98/53083 (ZENECA LIMITED) 26 November 1998	1-16,23,24,35,36,47-49,57 17-22,25-34,37-49,57

Form PCT/ISA/210 (continuation of Box C) (July 1998)

Printed: 22-04-2005 TIPAT 9991059

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 99/00195

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	98/53083	AU	74442/98
		GB	9710475

END OF ANNEX

Form PCT/ISA/210 (citation family annex) (July 1998)

007 20-04-2005

Printed: 22-04-2005 TIPAT 99910592

PCT/AU99/00195

REC'D 20 APR 1999
WIPO PCT

5

Patent Office
Canberra

I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PP 2492 for a patent by AG-GENE AUSTRALIA LTD and STATE OF QUEENSLAND THROUGH ITS DEPARTMENT OF PRIMARY INDUSTRIES filed on 20 March 1998.

EPO - DG 1
20. 04. 2005
(93)

WITNESS my hand this Fourteenth day of April 1999

KIM MARSHALL
MANAGER EXAMINATION SUPPORT AND SALES

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

Regulation 3.2

AUSTRALIAN
PROVISIONAL NO. DATE OF FILING
PP2492 20 MAR. 98
PATENT OFFICE

Ag-Gene Australia Ltd
AND
State of Queensland through its Department of Primary Industries

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Synthetic Genes and Genetic Constructs Comprising Same I"

The invention is described in the following statement:

909 20-04-2005

SYNTHETIC GENES AND GENETIC CONSTRUCTS COMPRISING SAME I

FIELD OF THE INVENTION

The present invention relates generally to synthetic genes for modifying endogenous gene expression in a cell, tissue or organ of a transgenic organism, in particular a transgenic animal or plant. More particularly, the present invention provides novel synthetic genes and genetic constructs which are capable of repressing delaying or otherwise reducing the expression of an endogenous gene or a target gene in an organism when introduced thereto.

BACKGROUND TO THE INVENTION

Controlling metabolic pathways in eukaryotic organisms is desirable for the purposes of producing novel traits therein or introducing novel traits into a particular cell, tissue or organ of said organism. Whilst recombinant DNA technology has provided significant progress in an understanding of the mechanisms regulating eukaryotic gene expression, much less progress has been made in the actual manipulation of gene expression to produce novel traits. Moreover, there are only limited means by which human intervention may lead to a repression, delay or reduction in eukaryotic gene expression.

Current methods for down-regulating gene expression using recombinant DNA technology comprise the introduction of a transgene to the cell which is capable of repressing expression of an endogenous target gene, either transcriptionally or post-transcriptionally. However, the precise mechanism is not known. Moreover, the efficiency of current approaches is low and the results are variable and unpredictable.

Attempts to improve the accuracy and predictability of methods for regulating gene expression in cells, in particular the repression, delay or reduction in expression of viral target genes in eukaryotic cells, foreign transgenes or other foreign genes introduced into cells, tissues or organs by natural means, or endogenous genes which are expressed to

Printed: 22-04-2005 TIPAT 99910592

produce undesirable traits for a particular purpose, have been largely unsuccessful possibly due to a lack of knowledge of the precise mechanisms involved. As a consequence, the efficiency of methods currently available remains low and highly variable.

In work leading up to the present invention, the inventors sought to elucidate the mechanisms involved in down-regulating gene expression in an attempt to provide improved methods therefor. In so doing the inventors have developed a wide range of synthetic genes capable of modulating gene expression in both prokaryotic and eukaryotic cells and genetic constructs comprising same.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description. Sequence identity numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined after the bibliography.

Throughout this specification and the claims that follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

SUMMARY OF THE INVENTION

The present invention provides novel synthetic genes and improved genetic constructs comprising same for modifying endogenous or target gene expression in cells, tissues and/or organs which are either transfected or stably transformed therewith.

SUB 20-04-2005

Printed: 22-04-2005 TIPA1 99910592

Accordingly, one aspect of the present invention provides a synthetic gene which is capable of modifying target gene expression in a cell, tissue or organ of a prokaryotic or eukaryotic organism which is transfected or transformed therewith, wherein said synthetic gene at least comprises a structural gene sequence comprising a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a derivative thereof or a complementary sequence thereto placed operably under the control of a promoter sequence which is operable in said cell, tissue or organ.

A further aspect of the invention provides a synthetic gene which is capable of modifying the expression of a target gene in a cell, tissue or organ of a prokaryotic or eukaryotic organism which is transfected or transformed therewith, wherein said synthetic gene at least comprises multiple structural gene sequences, wherein each of said structural gene sequences comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a derivative thereof or a complementary sequence thereto and wherein said multiple structural gene sequences are placed operably under the control of a single promoter sequence which is operable in said cell, tissue or organ.

A third aspect of the present invention provides a synthetic gene which is capable of modifying the expression of a target gene in a cell, tissue or organ of a prokaryote or eukaryote which is transfected or transformed therewith wherein said synthetic gene at least comprises multiple structural gene sequences wherein each of said structural gene sequences is placed operably under the control of a promoter sequence which is operable in said cell, tissue or organ and wherein each of said structural gene sequences comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a derivative thereof or a complementary sequence thereto.

A further aspect of the present invention provides a genetic construct which is capable of modifying the expression of an endogenous gene or target gene in a transformed or transfected cell, tissue or organ wherein said genetic construct at least comprises the synthetic gene of the invention and one or more origins of replication and/or selectable marker gene

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

sequences.

A still further aspect of the invention provides a cell, tissue, organ or organism comprising the synthetic genes and genetic constructs described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a copy of a diagrammatic representation of the plasmid pEGFP-N1 MCS.

Figure 2 is a copy of a diagrammatic representation of the plasmid pCMV.cass.

Figure 3 is a copy of a diagrammatic representation of the plasmid pCR.Bgl-GFP-Bam.

Figure 4 is a copy of a diagrammatic representation of the plasmid pCR.SV40L.

Figure 5 is a copy of a diagrammatic representation of the plasmid pCMV.SV40L.cass.

Figure 6 is a copy of a diagrammatic representation of the plasmid pCR.BEV.1.

Figure 7 is a copy of a diagrammatic representation of the plasmid pCR.BEV.2.

Figure 8 is a copy of a diagrammatic representation of the plasmid pCR.BEV.3.

Figure 9 is a copy of a diagrammatic representation of the plasmid pEGFP.BEV.1.

Figure 10 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.2.

Figure 11 is a copy of a diagrammatic representation of the plasmid pCMV.VEB.

Figure 12 is a copy of a diagrammatic representation of the plasmid pCMV.BEVnt.

313 20-04-2005

Figure 13 is a copy of a diagrammatic representation of the plasmid pCMV.BEVx2.

Figure 14 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.VEB.

Figure 15 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.GFP.VEB.

Figure 16 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.SV40L-0.

Figure 17 is a copy of a diagrammatic representation of the plasmid pCMV.0.SV40L.BEV.

Figure 18 is a copy of a diagrammatic representation of the plasmid pCMV.0.SV40L.VEB.

Figure 19 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.SV40L.BEV.

Figure 20 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.SV40L.VEB.

Figure 21 is a copy of a diagrammatic representation of the plasmid pCMV.SV40LR.cass.

Figure 22 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.SV40LR.

Figure 23 is a copy of a diagrammatic representation of the plasmid pCMV.TYR.

Figure 24 is a copy of a diagrammatic representation of the plasmid pCMV.TYRLIB.

Figure 25 is a copy of a diagrammatic representation of the plasmid pCMV.Lac.

Figure 26 is a copy of a diagrammatic representation of the plasmid pCMVLacI.OPRSV1.cass.

Figure 27 is a copy of a diagrammatic representation of the plasmid pCMVLacI.OPRSV1.GFP.cass.

Figure 28 is a copy of a diagrammatic representation of the plasmid pCMVLacI.TYR.OPRSV1.GFP.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the present invention provides a synthetic gene which is capable of modifying the expression of a target gene in a cell, tissue or organ wherein said synthetic gene at least comprises a structural gene comprising a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a derivative thereof or a complementary sequence thereto placed operably under the control of a promoter which is operable in said cell, tissue or organ.

Reference herein to a "gene" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences);

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) optionally comprising 5'- or 3'-untranslated sequences linked thereto; or

(iii) an amplified DNA fragment or other recombinant nucleic acid molecule produced *in vitro* and comprising all or a part of the coding region and/or 5'- or 3'-untranslated sequences linked thereto.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product, in particular a sense or antisense mRNA product or a peptide, oligopeptide or polypeptide or a biologically-active protein.

The term "synthetic gene" refers to a non-naturally occurring gene as hereinbefore defined which preferably comprises at least one or more transcriptional and/or translational regulatory sequences operably linked to a structural gene sequence.

The term "structural gene" shall be taken to refer to a nucleotide sequence which is capable of being transmitted to produce mRNA and optionally, encodes a peptide, oligopeptide, polypeptide or biologically active protein molecule. Those skilled in the art will be aware that not all mRNA is capable of being translated into a peptide, oligopeptide, polypeptide or protein, for example if the mRNA lacks a functional translation start signal or alternatively, if the mRNA is antisense mRNA. The present invention clearly encompasses synthetic genes comprising nucleotide sequences which are not capable of encoding peptides, oligopeptides, polypeptides or biologically-active proteins. In particular, the present inventors have found that such synthetic genes may be advantageous in modifying target gene expression in cells, tissues or organs of a prokaryotic or eukaryotic organism.

The term "target gene" shall be taken to refer to any gene, the expression of which is to be modified using the synthetic gene of the invention. Preferred target genes include, but are not limited to viral genes and foreign genes which have been introduced into the cell, tissue or organ or alternatively, genes which are endogenous to the cell, tissue or organ.

Wherein the target gene is a viral gene, it is particularly preferred that the viral gene encodes a function which is essential for replication or reproduction of the virus, such as but not limited to a DNA polymerase or RNA polymerase gene or a viral coat protein gene, amongst others. In a particularly preferred embodiment, the target gene comprises an RNA polymerase gene derived from a single-stranded (+) RNA virus such as bovine enterovirus (BEV), Sinbis alphavirus or a lentivirus such as, but not limited to, an immunodeficiency virus (eg. HIV-1) or alternatively, a DNA polymerase derived from a double-stranded DNA virus such as bovine herpesvirus or herpes simplex virus I (HSVI), amongst others.

Wherein the target gene is a foreign gene, those skilled in the art will be aware that it will have been introduced to the cell, tissue or organ using transformation technology or alternatively, comprise a gene derived from a pathogen which has been introduced to said cell, tissue or organ by naturally-occurring gene transfer processes.

Particularly preferred foreign target genes include any transgene which has been introduced to the cell, tissue or organ.

Wherein the target gene is a gene which is endogenous to the cell, tissue or organ, it is particular preferred that its expression is capable of being monitored by a visual assay, enzyme assay or immunoassay. Particularly preferred endogenous target genes are those detected by visual assay means.

The synthetic genes of the present invention may be derived from naturally-occurring genes by standard recombinant techniques, the only requirement being that the synthetic gene is substantially identical at the nucleotide sequence level to at least a part of the target gene, the expression of which is to be modified. By "substantially identical" is meant that the structural gene sequence of the synthetic gene is at least about 80% - 90% identical to 30 or more contiguous nucleotides of the target gene, more preferably at least about 90-95% identical to 30 or more contiguous nucleotides of the target gene and even more preferably at least about 95-99% identical or absolutely identical to 30 or ore contiguous nucleotides of the target gene.

Generally, a gene of the invention may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or additions without affecting its ability to modify target gene expression. Nucleotide insertional derivatives of the synthetic gene of the present invention include 5′ and 3′ terminal fusions as well as intra-sequence insertions of single or multiple nucleotides. Insertional nucleotide sequence variants are those in which one or more nucleotides are introduced into a predetermined site in the nucleotide sequence although random insertion is also possible with suitable screening of the resulting product.

Deletional variants are characterised by the removal of one or more nucleotides from the sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide inserted in its place. Such a substitution may be "silent" in that the substitution does not change the amino acid defined by the codon. Alternatively, substituents are designed to alter one amino acid for another similar acting amino acid, or amino acid of like charge, polarity, or hydrophobicity.

Accordingly, the present invention extends to homologues, analogues and derivatives of the synthetic genes described herein.

For the present purpose, "homologues" of a gene as hereinbefore defined or of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as the nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence within said sequence, of one or more nucleotide substitutions, insertions, deletions, or rearrangements.

"Analogues" of a gene as hereinbefore defined or of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as a nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence of any non-nucleotide constituents not normally present in said isolated nucleic acid molecule, for example carbohydrates, radiochemicals including radionucleotides, reporter molecules such as, but not limited to DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

"Derivatives" of a gene as hereinbefore defined or of a nucleotide sequence set forth herein shall be taken to refer to any isolated nucleic acid molecule which contains significant sequence similarity to said sequence or a part thereof. Generally, the nucleotide sequence of the present invention may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions. Nucleotide insertional derivatives of the nucleotide sequence of the present invention include 5′ and 3′ terminal fusions as well as

intra-sequence insertions of single or multiple nucleotides or nucleotide analogues. Insertional nucleotide sequence variants are those in which one or more nucleotides or nucleotide analogues are introduced into a predetermined site in the nucleotide sequence of said sequence, although random insertion is also possible with suitable screening of the resulting product being performed. Deletional variants are characterised by the removal of one or more nucleotides from the nucleotide sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide or nucleotide analogue inserted in its place.

Accordingly, the structural gene component of the synthetic gene may comprise a nucleotide sequence which is at least about 80% identical to at least about 30 contiguous nucleotides of an endogenous target gene, a foreign target gene or a viral target gene present in a cell, tissue or organ or a homologue, analogue, derivative thereof or a complementary sequence thereto.

Preferred structural gene components of the synthetic gene of the invention comprise at least about 20-30 nucleotides in length derived from a viral DNA polymerase, viral RNA polymerase, viral coat protein or visually-detectable gene, more particularly an RNA polymerase gene derived from a virus selected from the list comprising BEV, Sindbis alphavirus, HIV-1, bovine herpes virus and HSV1 or a visually-detectable gene which is involved in determining pigmentation, cell death or other external phenotype on a cell, tissue, organ or organism, amongst others.

In a particularly preferred embodiment, the structural gene component of the synthetic gene comprises at least about 20-30 nucleotides in length derived from the BEV RNA-dependent RNA polymerase gene or the murine tyrosinase gene or the *Escherichia coli lac* repressor gene *lacI* or a complementary sequence thereto.

The structural gene component may comprise a nucleotide sequence which encodes an amino acid sequence, with or without a translation start signal (ATG) or a nucleotide sequence which is complementary thereto. Those skilled in the art will be aware that, in the absence

of the translation start signal in an appropriate reading frame, the mRNA encoded by the structural gene will not be translated in most eukaryotic and prokaryotic cells.

Alternatively, the structural gene may comprise a nucleotide sequence which does not encode an amino acid sequence or more commonly, comprises one or more open reading frames which encode one or more peptides, oligopeptides or polypeptides which are unrelated at the amino acid sequence level to the amino acid sequence encoded by the target gene. For example, the mRNA product of the structural gene may be inserted into the synthetic gene of the invention so as to alter or disrupt the reading frame of the structural gene and produce one or more frame shift mutations in the translation product thereof relative to the translation product encoded by the target gene, notwithstanding a substantial identity between the structural gene and the target gene on the one hand and the corresponding mRNA products of the structural gene and the target gene on the other hand. Such effects may be produced by introducing one or two nucleotide substitutions or deletions into the structural gene, relative to the target gene sequence or alternatively, by introducing a translation start codon 5'-ATG-3' upstream of any nucleotide in the structural gene which occurs at a particular position in a codon of the corresponding target gene such that the position of said nucleotide in the codon of the structural gene is altered.

Alternatively, the structural gene may encode no amino acid sequence or one or more amino acid sequences which are unrelated to the amino acid sequence encoded by the target gene wherein said structural gene is transcribed in the antisense orientation from the synthetic gene promoter, relative to the direction of transcription of the corresponding target gene. In such circumstances, the mRNA product of the structural gene will comprise a nucleotide sequence which is complementary to the nucleotide sequence in the corresponding region of the mRNA encoded by the target gene.

The present invention clearly encompasses synthetic genes wherein the structural gene component is operably connected in the sense or antisense orientation to a promoter sequence and irrespective of the capacity of said structural gene to encode an amino acid sequence

which is encoded by the target gene. Accordingly, the structural gene component may further comprise 5'-untranslated region and/or 3'-untranslated region and/or intron (eg. SV40 intron) and/or a coding region derived from the target gene or a complementary nucleotide sequence thereto.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation in eukaryotic cells, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers). For expression in prokaryotic cells, such as bacteria, the promoter should at least contain the -35 box and -10 box sequences.

A promoter is usually, but not necessarily, positioned upstream or 5', of the structural gene component of the synthetic gene of the invention, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the structural gene.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of an isolated nucleic acid molecule, in a cell, such as a plant, animal, insect, fungal, yeast or bacterial cell. Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression of a structural gene which expression it regulates and/or to alter the spatial expression and/or temporal expression of same. For example, regulatory elements which confer inducibility on the expression of the structural gene may be placed adjacent to a heterologous promoter sequence driving expression of a nucleic acid molecule.

Placing a structural gene under the regulatory control of a promoter sequence means positioning said molecule such that expression is controlled by the promoter sequence. Promoters are generally positioned 5' (upstream) to the genes that they control. In the

construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in the synthetic genes of the present invention include viral, fungal, bacterial, animal and plant derived promoters capable of functioning in plant, animal, insect, fungal, yeast or bacterial cells. The promoter may regulate the expression of the structural gene component constitutively, or differentially with respect to cell, the tissue or organ in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as physiological stresses, or pathogens, or metal ions, amongst others.

Preferably, the promoter is capable of regulating expression of a nucleic acid molecule in a eukaryotic cell, tissue or organ, at least during the period of time over which the target gene is expressed therein and more preferably also immediately preceding the commencement of detectable expression of the target gene in said cell, tissue or organ.

Accordingly, strong constitutive promoters are particularly preferred for the purposes of the present invention or promoters which may be induced by virus infection or the commencement of target gene expression.

Examples of preferred promoters include the bacteriophage T7 promoter, bacteriophage T3 promoter, SP6 promoter, *lac* operator-promoter, *tac* promoter, SV40 late promoter, SV40 early promoter, RSV-LTR promoter, CMV IE promoter and the like.

Particularly preferred promoters contemplated herein include promoters operable in eukaryotic cells, for example the SV40 early promoter, SV40 late promoter or the CMV IE promoter sequence. Those skilled in the art will readily be aware of additional promoter sequences other than those specifically described.

In the present context, the terms "in operable connection with" or "operably under the control" or similar shall be taken to indicate that expression of the structural gene is under the control of the promoter sequence with which it is spatially connected; in a cell, tissue, organ or whole organism.

In a more particularly preferred embodiment of the invention, the synthetic gene according to this aspect of the invention comprises the coding region of the BEV polymerase gene placed in the sense orientation operably under the control of the CMV IE promoter or SV40 late promoter. In an alternative embodiment, the synthetic gene comprises a nucleotide sequence derived from the coding region of the BEV polymerase gene but lacking a translation - start site, placed in the sense orientation in operable connection with the CMV IE promoter or SV40 late promoter. In a further alternative embodiment, the synthetic gene comprises a nucleotide sequence derived from the BEV polymerase gene placed in the antisense orientation relative to the BEV polymerase gene and in operable connection with the CMV IE promoter or the SV40 late promoter sequence.

For the present purposes, the term "BEV polymerase" as used herein shall be taken to refer to a structural gene, cDNA molecule, genomic gene or nucleotide sequence at least about 30-50 nucleotides in length which is derived from the nucleotide sequence of the bovine enterovirus(BEV) RNA-dependent RNA polymerase gene, including both translatable and non-translatable nucleotide sequences and nucleotide sequences which are complementary to a part of the nucleotide sequence of the full-length BEV RNA-dependent RNA polymerase gene.

In a further alternative embodiment, the synthetic gene according to this aspect of the

invention comprises the coding region of a tyrosinase gene, in particular the murine tyrosinase gene, placed in the sense orientation operably under the control of the CMV IE promoter or SV40 late promoter. As with other embodiments described herein, the synthetic gene (i.e. tyrosinase gene) may lack a functional translation start site or be introduced in the antisense orientation. The present invention clearly encompasses all such embodiments.

As used herein, the term "tyrosinase gene" shall be taken to refer to a structural gene, cDNA molecule, genomic gene or nucleotide sequence which is capable of encoding the tyrosinase enzyme or a polypeptide fragment thereof or alternatively, a nucleotide sequence which is complementary to said structural gene, cDNA molecule, genomic gene or nucleotide sequence. Particularly preferred tyrosinase genes for use in the performance of the present invention include, but are not limited to, those described by Kwon *et al* (1988) and homologues, analogues and derivatives thereof and complementary nucleotide sequences thereto.

In still a further alternative embodiment, the synthetic gene according to this aspect of the invention comprises the coding region of the *lac*I gene, placed in the sense orientation operably under the control of the CMV IE promoter or SV40 late promoter. As with other embodiments described herein, the synthetic gene (i.e. *E. coli lac*I gene) may lack a functional translation start site or be introduced in the antisense orientation. The present invention clearly encompasses all such embodiments.

As used herein, the term "*lac*I gene" shall be taken to refer to a structural gene, cDNA molecule, genomic gene or nucleotide sequence which is capable of encoding a polypeptide repressor of the *lac*Z gene which encodes the enzyme β-galactosidase or alternatively, a nucleotide sequence which is complementary to said structural gene, cDNA molecule, genomic gene or nucleotide sequence. Those skilled in the art will be aware that the *lac* repressor is a DNA-binding protein which acts on the *lac* operator-promoter sequence. In the presence of one of a variety of β-galactosides, the affinity of the *lac* repressor for the *lac* operator-promoter sequence is lowered, thereby allowing RNA polymerase to bind the *lac*

Printed: 22-04-2005 TIPAT 99910592

P:\OPER\MROAG-GENEI.PRV - 19/3/98

operator-promoter region to activate transcription of the *lac* operon.

Standard methods may be used to produce the structural genes of the present invention, in particular the BEV polymerase and tyrosinase genes which are derived from publicly available material. For example, the BEV polymerase and tyrosinase genes may be amplified using the polymerase chain reaction or alternatively, isolated using standard hybridisation techniques known to those skilled in the art.

For the purposes of nomenclature, the nucleotide sequence of the cDNA encoding murine tyrosinase is publicly available under GenBank Accession No. M20234.

A second aspect of the present invention provides a synthetic gene which is capable of modifying the expression of a target gene in a cell, tissue or organ, wherein said synthetic gene at least comprises multiple structural gene sequences wherein each of said structural gene sequences comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of the target gene or a derivative thereof or a complementary sequence thereto and wherein said multiple structural gene sequences are placed operably under the control of a single promoter sequence which is operable in said cell, tissue or organ.

As used herein, the term "multiple structural gene sequences" or similar term shall be taken to refer to any number of structural genes as defined herein which is greater than or equal to two. Accordingly, a multiple structural gene sequence may comprise a tandem repeat or concatemer of two or more identical nucleotide sequences or alternatively, a tandem array or concatemer of non-identical nucleotide sequences, the only requirement being that each of the structural gene sequences contained therein is substantially identical to the target gene sequence or a complementary sequence thereto. In this regard, those skilled in the art will be aware that a cDNA molecule may also be regarded as a multiple structural gene sequence in the context of the present invention, in so far as it comprises a tandem array or concatemer of exon sequences derived from a genomic target gene. Accordingly, cDNA molecules and any tandem array, tandem repeat or concatemer of exon sequences and/or intron sequences

325 20-04-2005

Printed: 22-04-2005 IPA 99910592

and/or 5'-untranslated and/or 3'-untranslated sequences are clearly encompassed by this embodiment of the invention.

Preferably, the multiple structural gene comprises at least 2-4 individual structural gene sequences, more preferably at least about 4-6 individual structural gene sequences and more preferably at least about 6-8 individual structural gene sequences.

The optimum number of structural gene sequences which may be involved in the synthetic gene of the present invention will vary considerably, depending upon the length of each of said structural gene sequences, their orientation and degree of identity to each other. For example, those skilled in the art will be aware of the inherent instability of palindromic nucleotide sequences *in vivo* and the difficulties associated with constructing long synthetic genes comprising inverted repeated nucleotide sequences, because of the tendency for such sequences to form hairpin loops and to recombine *in vivo*. Notwithstanding such difficulties, the optimum number of structural gene sequences to be included in the synthetic genes of the present invention may be determined empirically by those skilled in the art, without any undue experimentation and by following standard procedures such as the construction of the synthetic gene of the invention using recombinase-deficient cell lines, reducing the number of repeated sequences to a level which eliminates or minimises recombination events and by keeping the total length of the multiple structural gene sequence to an acceptable limit, preferably no more than 5-10kb, more preferably no more than 2-5kb and even more preferably no more than 0.5-2.0kb in length.

In an alternative embodiment, each structural gene contained within the multiple structural gene unit of the subject synthetic gene may comprise a nucleotide sequence which is substantially identical to a different target gene in the same organism. Such an arrangement may be of particular utility when the synthetic gene is intended to provide protection against a pathogen in a cell, tissue or organ, in particular a viral pathogen, by modifying the expression of viral target genes. For example, the multiple structural gene may comprise nucleotide sequences which are substantially identical to two or more target genes selected

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

from the list comprising DNA polymerase, RNA polymerase and coat protein or other target gene which is essential for viral infectivity, replication or reproduction. However, it is preferred with this arrangement that the structural gene units are selected such that the target genes to which they are substantially identical are normally expressed at approximately the same time (or later) in an infected cell, tissue or organ as (than) the multiple structural gene of the subject synthetic gene is expressed under control of the promoter sequence. This means that the promoter controlling expression of the multiple structural gene will usually be selected to confer expression in the cell, tissue or organ over the entire life cycle of the virus when the viral target genes are expressed at different stages of infection.

The individual structural gene units of the multiple structural gene according to the embodiments described herein may be spatially connected in any orientation relative to each other, for example head-to-head, head-to-tail or tail-to-tail and all such configurations are within the scope of the invention.

Preferably, the multiple structural gene unit comprises two structural genes in a head-to-tail or head-to-head configuration. More preferably, the multiple structural gene unit comprises two identical or substantially identical structural genes or a homologue, analogue or derivative thereof in a head-to-tail configuration as a direct repeat or alternatively, in a head-to-head configuration as an inverted repeat or palindrome.

In a particularly preferred embodiment, the multiple structural gene unit comprises two identical or substantially identical structural genes comprising nucleotide sequences derived from the BEV polymerase or tyrosinase gene or a homologue, analogue or derivative thereof, placed in a head-to-head or head-to-tail configuration.

According to this aspect of the invention, wherein the multiple structural gene or any individual structural gene unit thereof is intended to be both transcribed and translated, a translation start signal may be included at the 5′ end of that open reading frame. In a particularly preferred embodiment, the structural gene unit which is positioned nearer the 5′

327 20-04-2005

end of the synthetic gene comprises an in-frame translation start signal of facilitate translation of the first open reading frame of the multiple structural gene in a cell, tissue or organ into which the synthetic gene is introduced. Those skilled in the art will be aware that it is also possible to produce a fusion polypeptide from such an arrangement provided that the individual structural gene units are positioned such that their open reading frames are in-frame with respect to each other or alternatively, the individual structural gene units are separated by intron/exon splice boundary sequences such that splicing of the mRNA product of the synthetic gene produces a translatable mRNA wherein the said open reading frames are in-frame with respect to each other. Such embodiments are clearly contemplated by the present invention. Intron/exon splice junction sequences are well-known in the art and the skilled person would readily be able to introduce such sequences to the 5'- and 3'- ends of a structural gene unit of the synthetic genes described herein.

The individual structural genes comprising the multiple structural gene unit may be further spatially separated by the addition of a linker molecule or "stuffer fragment" there between. The stuffer fragment may comprise any combination of nucleotide or amino acid residues, carbohydrate molecules or oligosaccharide molecules or carbon atoms or a homologue, analogue or derivative thereof which is capable of being linked covalently to a nucleic acid molecule.

Preferably, embodiment, the stuffer fragment comprises a sequence of nucleotides or a homologue, analogue or derivative thereof.

More preferably, the stuffer fragment comprises a sequence of nucleotides of at least about 10-50 nucleotides in length, even more preferably at least about 50-100 nucleotides in length and still more preferably at least about 100-500 nucleotides in length.

Wherein the multiple structural gene unit comprises intron/exon splice junction sequences, the stuffer fragment may serve as an intron sequence placed between the 3'-splice site of the structural gene nearer the 5'-end of the gene and the 5'- splice site of the next downstream

Printed: 22-04-2005 TIPAT 99910592

structural gene. Alternatively, wherein it is desirable for more than two adjacent structural genes to be translated, the stuffer fragment placed there between should not include an in-frame translation stop codon, absent intron/exon splice junction sequences at both ends of the stuffer fragment or the addition of a translation start codon at the 5′ end of each structural gene unit, as will be obvious to those skilled in the art.

Preferred stuffer fragments are those which encode detectable marker proteins or biologically-active analogues and derivatives thereof, for example luciferase, β-galacturonase, β-galactosidase, chloramphenicol acetyltransferase or green fluorescent protein, amongst others.

According to this embodiment, the detectable marker or an analogue or derivative thereof serves to indicate the expression of the synthetic gene of the invention in a cell, tissue or organ by virtue of its ability to confer a specific detectable phenotype thereon, preferably a visually-detectable phenotype.

In a more particularly preferred embodiment of the invention, the multiple structural gene comprises an interrupted direct repeat or interrupted palindrome comprising two identical or substantially-identical BEV polymerase structural gene sequences or alternatively, two identical or substantially-identical tyrosinase structural gene sequences or a homologue, analogue or derivative thereof separated by a stuffer fragment comprising a nucleotide sequence which encodes green-fluorescent protein or a biologically-active analogue or derivative thereof.

As used herein, the term "green fluorescent protein" or "GFP" shall be taken to refer to a protein, polypeptide or peptide which is capable of producing a strong green fluorescence when excited with near ultraviolet radiation or blue light or a homologue, analogue or derivative thereof. Accordingly, the term "GFP gene" shall be taken to refer to a nucleotide sequence which is capable of encoding GFP or a complementary nucleotide sequence thereto. Particularly preferred GFPs and GFP genes according to the present invention are derived from the jellyfish *Aequoria victoria* as described by Prasher *et al* (1992) or in International

529 20-04-2005

Printed: 22-04-2005 IPAU 99910592

Patent Publication No. WO 95/07463, amongst others.

A further aspect of the invention provides for each structural gene of the multiple structural gene unit to be placed operably under the control of a separate promoter sequence.

According to this embodiment, the promoters controlling expression of the structural gene unit are preferably different promoter sequences, to reduce competition there between for cellular transcription factors and RNA polymerases. Preferred promoters are selected from those referred to *supra*.

Those skilled in the art will know how to modify the arrangement or configuration of the individual structural genes as described *supra* to regulate their expression from separate promoter sequences.

In a particularly preferred embodiment, the multiple structural gene unit comprises two or more BEV polymerase structural genes or two or more tyrosinase structural genes wherein each of said structural genes is placed operably in connection with a different promoter sequence. More particularly preferred, the multiple structural gene unit comprises two BEV polymerase structural genes or two tyrosinase structural genes positioned as inverted repeats or direct repeats wherein one of said structural genes is placed operably in connection with the CMV IE promoter. Even more preferably, at least one of the BEV polymerase structural genes or tyrosinase genes comprising the multiple structural gene is presented in the sense orientation and comprises a translation start signal to facilitate translation of mRNA encoded therefrom.

Those skilled in the art will be aware that the structural genes comprising the multiple structural gene unit according to this aspect of the invention are expressed as physically-distinct mRNA species and, as a consequence, wherein said mRNA species are translated, no fusion polypeptide will be produced there between. However, the present invention clearly extends to synthetic gene which comprises two or more structural genes operably connected

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

to a first promoter sequence and one or more structural genes operably connected to one or more additional promoter sequences.

The synthetic genes described *supra* are capable of being modified further, for example by the inclusion of marker nucleotide sequences encoding a detectable marker enzyme or a functional analogue or derivative thereof, to facilitate detection of the synthetic gene in a cell, tissue or organ in which it is expressed. According to this embodiment, the marker nucleotide sequences will be present in a translatable format and expressed, for example as a fusion polypeptide with the translation product(s) of any one or more of the structural genes or alternatively as a non-fusion polypeptide.

Alternatively or in addition, the synthetic genes described *supra* may further comprise one or more transcription termination sequences placed at the 3'-end of the transcriptional unit of the synthetic gene sequence.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

Examples of terminators particularly suitable for use in the synthetic genes of the present invention include the SV40 polyadenylation signal, the HSV TK polyadenylation signal, the CYC1 terminator, ADH terminator, SPA terminator, nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens*, the terminator of the Cauliflower mosaic virus (CaMV) 35S gene, the *zein* gene terminator from *Zea mays*, the Rubisco small subunit gene (SSU) gene terminator sequences, subclover stunt virus (SCSV) gene sequence terminators, any *rho*-independent *E.coli* terminator, or the *lacZ* alpha terminator, amongst others.

BSDOCID: 20-04-2005

In a particularly preferred embodiment, the terminator is the SV40 polyadenylation signal or the HSV TK polyadenylation signal which are operable in animal cells, tissues and organs or the *lacZ* alpha terminator which is active in prokaryotic cells.

Those skilled in the art will be aware of additional promoter sequences and terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

The synthetic genes of the present invention may be introduced to a suitable cell, tissue or organ without modification as linear DNA in the form of a genetic construct, optionally contained within a suitable carrier, such as a cell, virus particle or liposome, amongst others. To produce a genetic construct, the synthetic gene of the invention is inserted into a suitable vector or episome molecule, such as a bacteriophage vector, viral vector or a plasmid, cosmid or artificial chromosome vector which is capable of being maintained and/or replicated and/or expressed in the host cell, tissue or organ into which it is subsequently introduced.

Accordingly a further aspect of the invention provides a genetic construct which at least comprises the synthetic gene according to any one or more of the embodiments described herein and one or more origins of replication and/or selectable marker gene sequences.

Usually, an origin of replication or a selectable marker gene suitable for use in bacteria is physically-separated from those genetic sequences contained in the genetic construct which are intended to be expressed or transferred to a eukaryotic cell, or integrated into the genome of a eukaryotic cell.

In a particularly preferred embodiment, the origin of replication is functional in a bacterial cell and comprises the pUC or the ColE1 origin or alternatively the origin of replication is operable in a eukaryotic cell, tissue and more preferably comprises the 2 micron (2μm) origin of replication or the SV40 origin of replication.

Printed: 22-04-2005 IPAU 9910592

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a genetic construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin-resistance gene (Amp^r), tetracycline-resistance gene (Tc^r), bacterial kanamycin-resistance gene (Kan^r), is the zeocin resistance gene (Zeocin is a drug of bleomycin family which is trademark of InVitrogen Corporation), the *AURI-C* gene which confers resistance to the antibiotic aureobasidin A, phosphinothricin-resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin-resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein- encoding gene or the luciferase gene, amongst others.

Preferably, the selectable marker gene is the *npt*II gene or Kan^r gene or green fluorescent protein (GFP)-encoding gene.

Those skilled in the art will be aware of other selectable marker genes useful in the performance of the present invention and the subject invention is not limited by the nature of the selectable marker gene.

The present invention extends to all genetic constructs essentially as defined herein, which include further genetic sequences intended for the maintenance and/or replication of said genetic construct in prokaryotes and/or the integration of said genetic construct or a part thereof into the genome of a eukaryotic cell or organism.

The present invention further extends to an isolated cell, tissue or organ comprising the synthetic gene described herein or a genetic construct comprising same. Any standard means may be used for their introduction including cell mating, transformation or transfection procedures known to those skilled in the art or described by Ausubel *et al.* (1992).

6868 20-04-2005

The present invention is further described by reference to the following non-limiting Examples.

EXAMPLE 1

Base Plasmids

Plasmid pEGFP-N1 MCS

Plasmid pEGFP-N1 MCS (Figure 1; Clontech) contains the CMV IE promoter operably connected to an open reading frame encoding a red-shifted variant of wild-type green fluorescent protein (GFP; Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Inouye and Tsuji, 1994), which has been optimised for brighter fluorescence. The specific GFP variant encoded by pEGFP-N1 MCS has been disclosed by Cormack *et al.* (1996). Plasmid pEGFP-N1 MCS contains a multiple cloning site comprising *Bgl*II and *Bam*HI sites and many other restriction endonuclease cleavage sites, located between the CMV IE promoter and the GFP open reading frame. Structural genes cloned into the multiple cloning site will be expressed at the transcriptional level if they lack a functional translation start site, however such structural gene sequences will not be expressed at the protein level (i.e. translated). Structural gene sequences inserted into the multiple cloning site which comprise a functional translation start site will be expressed as GFP fusion polypeptides if they are cloned in-frame with the GFP-encoding sequence. The plasmid further comprises an SV40 polyadenylation signal downstream of the GFP open reading frame to direct proper processing of the 3'-end of mRNA transcribed from the CMV-IE promoter sequence. The plasmid further comprises the SV40 origin of replication functional in animal cells; the neomycin-resistance gene comprising SV40 early promoter (SV40 EP in Figure 1) operably connected to the neomycin/kanamycin-resistance gene derived from Tn5 (Kan/neo in Figure 1) and the HSV thymidine kinase polyadenylation signal (HSV TK poly (A) in Figure 1), for selection of transformed cells on kamanycin, neomycin or G418; the pUC19 origin of replication which is functional in bacterial cells (pUC Ori in Figure 1); and the f1 origin of replication for

Printed: 22-04-2005 TIPA 99910592

single-stranded DNA production (f1 Ori in Figure 1).

pCMVLacI

Plasmid pCMVLacI is a commercially-obtainable mammalian expression vector (Stratagene) comprising the *lac*I gene encoding the *lac* repressor and a gene coding for hygromycin resistance (Hyg^r).

Plasmid pOPRSVI/MCS

Plasmid pOPRSVI/MCS is a commercially-obtainable mammalian expression vector (Stratagene), comprising the OPRSV1 promoter sequence (a modified RSV-LTR promoter), SV40 intron sequence, *lac* operator sequence, multiple cloning site and thymidine kinase (TK) gene transcription terminator sequence [i.e. TK poly(A) signal].

Plasmid pSVL

Plasmid pSVL is commercially-obtainable from Pharmacia and serves as a source of the SV40 late promoter sequence. The nucleotide sequence of pSVL is also publicly available as GenBank Accession Number U13868.

Plasmid pCMV.cass

Plasmid pCMV.cass (Figure 2) is an expression cassette for driving expression of a structural gene sequence under control of the CMV-IE promoter sequence. Plasmid pCMV.cass was derived from pEGFP-N1 MCS by deletion of the GFP open reading frame as follows: Plasmid pEGFP-N1 MCS was digested with *Pin*AI and *Not* I, blunt-ended using *Pfu*I polymerase and then re-ligated. Structural gene sequences are cloned into pCMV.cass using the multiple cloning site, which is identical to the multiple cloning site of pEGFP-N1 MCS, except it lacks the *Pin*AI site.

385 20-04-2005

Plasmid pCR2.1

Plasmid pCR2.1 is commercially available from Stratagene and comprises the lacZ promoter sequence and *lacZ*-α transcription terminator, with a cloning site for the insertion of structural gene sequences there between. Plasmid pCR2.1 is designed to clone nucleic acid fragments by virtue of the A-overhang frequently synthesized by *Taq* polymerase during the polymerase chain reaction. The plasmid further comprises the ColE1 and f1 origins of replication and kanamycin-resistance and ampicillin-resistance genes.

Plasmid pCR.Bgl-GFP-Bam

Plasmid pCR.Bgl-GFP-Bam (Figure 3) comprises an internal region of the GFP open reading frame derived from plasmid pEGFP-N1 MCS (Figure 1) placed operably under the control of the lacZ promoter. To produce this plasmid, a region of the GFP open reading frame was amplified from pEGFP-N1 MCS using the amplification primers Bgl-GFP (SEQ ID NO:5) and GFP-Bam (SEQ ID NO:6) and cloned into plasmid pCR2.1. The internal GFP-encoding region in plasmid pCR.Bgl-GFP-Bam lacks functional translational start and stop codons.

Plasmid pCR.SV40L

Plasmid pCR.SV40L (Figure 4) comprises the SV40 late promoter derived from plasmid pSVL (GenBank Accession No. U13868; Pharmacia), cloned into pCR2.1 (Stratagene). To produce this plasmid, the SV40 late promoter was amplified using the primers SV40-1 (SEQ ID NO:7) and SV40-2 (SEQ ID NO:8) which comprise *Sal* I cloning sites to facilitate sub-cloning of the amplified DNA fragment into pCMV.cass. SEQ ID No. 7 also contains a synthetic poly (A) site at the 5′ end, such that the amplicification product comprises the synthetic poly(A) site at the 5′ end of the SV40 promoter sequence.

Plasmid pCMV.SV40L.cass

Plasmid pCMV.SV40L.cass (Figure 5) comprises the synthetic poly A site and the SV40 late promoter sequence from plasmid pCR.SV40L (Figure 4), sub-cloned as a *Sal* I fragment, into the *Sal* I site of plasmid pCMV.cass (Figure 2), such that the CMV-IE promoter and SV40 late promoter sequences are capable of directing transcription in the same direction. Accordingly, the synthetic poly(A) site at the 5′ end of the SV40 promoter sequence is used as a transcription terminator for structural genes expressed from the CMV IE promoter in this plasmid, which also provides for the insertion of said structural gene via the multiple cloning site present between the SV40 late promoter and the synthetic poly(A) site (Figure 5). The multiple cloning sites are located behind the CMV-IE and SV40 late promoters, including *Bam*HI and *Bgl*II sites.

EXAMPLE 2

BEV Polymerase-containing genes

Plasmid pCR.BEV.1

The BEV RNA-dependent RNA polymerase coding region was amplified as a 1,385 bp DNA fragment from a full-length cDNA clone encoding same, using the primers designated BEV-1 (SEQ ID NO:1) and BEV-2 (SEQ ID NO:2), under standard amplification conditions. The amplified DNA contained a 5′-*Bgl* II restriction enzyme site, derived from the BEV-1 primer sequence and a 3′*Bam*HI restriction enzyme site, derived from the BEV-2 primer sequence. Additionally, as the BEV-1 primer sequence contains a translation start signal 5′-ATG-3′ engineered at positions 15-17 of SEQ ID NO:1, the amplified BEV polymerase structural gene comprises the start site in-frame with BEV polymerase-encoding nucleotide sequences, Thus, the amplified BEV polymerase structural gene comprises the ATG start codon immediately upstream (ie. juxtaposed) to the BEV polymerase-encoding sequence. There is no translation stop codon in the amplified DNA.

Printed: 22-04-2005 TIPAT 99910592

The amplified BEV polymerase structural gene was cloned into plasmid pCR2.1 to produce pCR.BEV.1 (Figure 6).

Plasmid pCR.BEV.2

The complete BEV polymerase coding region was amplified from a full-length cDNA clone encoding same, using primers BEV-1 (SEQ ID NO:1) and BEV-3 (SEQ ID NO:3). Primer BEV-3 comprises a *Bam*HI restriction enzyme site at positions 5 to 10 inclusive of SEQ ID NO:3 and the complement of a translation stop signal at positions 11 to 13 of SEQ ID NO:3. As a consequence, an open reading frame comprising a translation start signal and translation stop signal, contained between the *Bgl* II and *Bam*HI restriction sites. The amplified fragment was cloned into pCR2.1 to produce plasmid pCR2.BEV.2 (Figure 7).

Plasmid pCR.BEV.3

A non-translatable BEV polymerase structural gene was amplified from a full-length BEV polymerase cDNA clone using the amplification primers BEV-3 (SEQ ID NO:3) and BEV-4 (SEQ ID NO:4). Primer BEV-4 comprises a *Bgl*II cloning site at positions 5-10 of SEQ ID NO:4 and sequences downstream of this *Bgl*II site are homologous to nucleotide sequences of the BEV polymerase gene. There is no functional ATG start codon in the amplified DNA product of primers BEV-3 and BEV-4. The BEV polymerase is expressed as part of a polyprotein and, as a consequence, there is no ATG translation start site in this gene. The amplified DNA was cloned into plasmid pCR2.1 to yield plasmid pCR.BEV.3 (Figure 8).

398 20-04-2005

EXAMPLE 3

Synthetic genes comprising a BEV polymerase structural gene operably connected to the CMV-IE promoter sequence

Plasmid pEGFP.BEV.1

Plasmid pEGFP.BEV.1 (Figure 9) is capable of expressing the BEV polymerase structural gene as a GFP fusion polypeptide under the control of the CMV-IE promoter sequence. To produce plasmid pEGFP.BEV.1, the BEV polymerase sequence from pCR.BEV.1 (Figure 6) was cloned as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pEGFP-N1 MCS (Figure 1).

Plasmid pCMV.BEV.2

Plasmid pCMV.BEV.2 (Figure 10) is capable of expressing the entire BEV polymerase open reading frame under the control of CMV-IE promoter sequence. To produce pCMV.BEV.2, the BEV polymerase sequence from pCR.BEV.2 (Figure 7) was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.VEB

Plasmid pCMV.VEB (Figure 11) expresses an antisense BEV polymerase mRNA under the control of the CMV-IE promoter sequence. To produce plasmid pCMV.VEB, the BEV polymerase sequence from pCR.BEV.2 (Figure 7) was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.BEVnt

Plasmid pCMV.BEVnt (Figure 12) expresses a non-translatable BEV polymerase structural gene in the sense orientation under the control of the CMV-IE promoter sequence. To

produce pCMV.BEVnt, the BEV polymerase sequence from pCR.BEV.3 (Figure 8) was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.BEVx2

Plasmid pCMV.BEVx2 (Figure 13) comprises a direct repeat of a complete BEV polymerase open reading frame under the control of the CMV-IE promoter sequence. In eukaryotic cells at least, the open reading frame located nearer the CMV-IE promoter is translatable. To produce pCMV.BEVx2, the BEV polymerase structural gene from plasmid pCR.BEV.2 (Figure 7) was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.2 (Figure 10), immediately downstream of the BEV polymerase structural gene already present therein.

Plasmid pCMV.BEV.VEB

Plasmid pCMV.BEV.VEB (Figure 14) comprises an inverted repeat or palindrome of a complete BEV polymerase open reading frame under the control of the CMV-IE promoter sequence. In eukaryotic cells at least, the open reading frame located nearer the CMV-IE promoter is translatable. To produce pCMV.BEV.VEB, the BEV polymerase structural gene from plasmid pCR.BEV.2 (Figure 7) was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.2 (Figure 10), immediately downstream of the BEV polymerase structural gene already present therein.

Plasmid pCMV.BEV.GFP.VEB

Plasmid pCMV.BEV.GFP.VEB (Figure 15) is similar to plasmid pCMV.BEV.VEB except that the BEV structural gene inverted repeat or palindrome is interrupted by the insertion of a GFP open reading frame (stuffer fragment) therein. To produce plasmid pCMV.BEV.GFP.VEB, the GFP stuffer fragment from pCR.Bgl-GFP-Bam (Figure 3) was

Printed: 22-04-2005 IIPAT 99910592

first sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.2 (Figure 7) to produce an intermediate plasmid pCMV.BEV.GFP wherein the BEV polymerase-encoding and GFP-encoding sequences are contained within the same 5'-*Bgl*II-to-*Bam*HI-3' fragment. The BEV polymerase structural gene from pCMV.BEV.2 (Figure 7) was then cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.GFP. The BEV polymerase structural gene nearer the CMV-IE promoter sequence in plasmid pCMV.BEV.GFP.VEB is capable of being translated, at least in eukaryotic cells.

EXAMPLE 4

Synthetic genes comprising BEV polymerase structural genes operably connected to multiple promoter sequences

Plasmid pCMV.BEV.SV40L-O

Plasmid pCMV.BEV.SV40L-O (Figure 16) comprises a translatable BEV polymerase structural gene derived from plasmid pCR.BEV.2 (Figure 7) inserted in the sense orientation between the CMV-IE promoter and the SV40 late promoter sequences of plasmid pCMV.SV40L.cass (Figure 5). To produce plasmid pCMV.BEV.SV40L-O, the BEV polymerase structural gene was sub-cloned as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II-digested pCMV.SV40L.cass DNA.

Plasmid pCMV.O.SV40L.BEV

Plasmid pCMV.O.SV40L.BEV (Figure 17) comprises a translatable BEV polymerase structural gene derived from plasmid pCR.BEV.2 (Figure 7) cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences present in plasmid pCMV.SV40L.cass (Figure 5). To produce plasmid pCMV.O.SV40L.BEV, the BEV polymerase structural gene was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.SV40L.cass DNA.

20-04-2005

Plasmid pCMV.O.SV40L.VEB

Plasmid pCMV.O.SV40L.VEB (Figure 18) comprises an antisense BEV polymerase structural gene derived from plasmid pCR.BEV.2 (Figure 7) cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences present in plasmid pCMV.SV40L.cass (Figure 5). To produce plasmid pCMV.O.SV40L.VEB, the BEV polymerase structural gene was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.SV40L.cass DNA.

Plasmid pCMV.BEV.SV40L.BEV

Plasmid pCMV.BEV.SV40L.BEV(Figure 19) comprises a multiple structural gene unit comprising two BEV polymerase structural genes placed operably and separately under control of the CMV-IE promoter and SV40 late promoter sequences. To produce plasmid pCMV.BEV.SV40L.BEV, the translatable BEV polymerase structural gene present in pCR.BEV.2 (Figure 7) was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment behind the SV40 late promoter sequence present in *Bam*HI-digested pCMV.BEV.SV40L-O (Figure 16).

Plasmid pCMV.BEV.SV40L.VEB

Plasmid pCMV.BEV.SV40L.VEB (Figure 20) comprises a multiple structural gene unit comprising two BEV polymerase structural genes placed operably and separately under control of the CMV-IE promoter and SV40 late promoter sequences. To produce plasmid pCMV.BEV.SV40L.VEB, the translatable BEV polymerase structural gene present in pCR.BEV.2 (Figure 7) was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment behind the SV40 late promoter sequence present in *Bam*HI-digested pCMV.BEV.SV40L-O (Figure 16). In this plasmid, the BEV polymerase structural gene is expressed in the sense orientation under control of the CMV-IE promoter to produce a translatable mRNA, whilst the BEV polymerase structural gene is also expressed under control of the SV40 promoter to produce an antisense mRNA species.

Plasmid pCMV.SV40LR.cass

Plasmid pCMV.SV40LR.cass (Figure 21) comprises the SV40 late promoter sequence derived from plasmid pCR.SV40L (Figure 4), sub-cloned as a *SalI* fragment into the *SalI* site of the plasmid pCMV.cass (Figure 2), such that the CMV-IE or the SV40 late promoter may drive transcription of a structural gene or a multiple structural gene unit, in the sense or antisense orientation, as desired. A multiple cloning site is positioned between the opposing CMV- IE and SV40 late promoter sequences in this plasmid to facilitate the introduction of a structural gene sequence. In order for expression of a structural gene sequence to occur from this plasmid, it must be introduced with its own transcription termination sequence located at the 3′ end, because there are no transcription termination sequences located between the opposing CMV- IE and SV40 late promoter sequences in this plasmid. Preferably, the structural gene sequence or multiple structural gene unit which is to be introduced into pCMV.SV40LR.cass will comprise both a 5′ and a 3′ polyadenylation signal as follows:

(i) where the structural gene sequence or multiple structural gene unit is to be expressed in the sense orientation from the CMV IE promoter sequence and/or in the antisense orientation from the SV40 late promoter, the 5′ polyadenylation signal will be in the antisense orientation and the 3′ polyadenylation signal will be in the sense orientation; and

(ii) where the structural gene sequence or multiple structural gene unit is to be expressed in the antisense orientation from the CMV IE promoter sequence and/or in the sense orientation from the SV40 late promoter, the 5′ polyadenylation signal will be in the sense orientation and the 3′ polyadenylation signal will be in the antisense orientation.

Alternatively or in addition, suitably-oriented terminator sequences may be placed at the 5′-end of the CMV and SV40L promoters, as shown in Figure 21.

Alternatively, plasmid pCMV.SV40LR.cass is further modified to produce a derivative plasmid which comprises two polyadenylation signals located between the CMV IE and SV40

late promoter sequences, in approriate orientations to facilitate expression of any structural gene located therebetween in the sense or antisense orientation from either the CMV IE promoter or the SV40 promoter sequence. The present invention clearly encompasses such derivatives.

Plasmid pCMV.BEV.SV40LR

Plasmid pCMV.BEV.SV40LR (Figure 22) comprises a structural gene comprising the entire BEV polymerase open reading frame placed operably and separately under control of opposing CMV-IE promoter and SV40 late promoter sequences, thereby potentially producing BEV polymerase transcripts at least from both strands of the full-length BEV polymerase structural gene. To produce plasmid pCMV.BEV.SV40LR, the translatable BEV polymerase structural gene present in pCR.BEV.2 (Figure 7) was sub-cloned, as a *Bgl*II-to-*Bam*HI fragment, into the unique *Bgl*II site of plasmid pCMV.SV40LR.cass (Figure 21), such that the BEV open reading frame is present in the sense orientation relative to the CMV-IE promoter sequence.

Those skilled in the art will recognise that it is possible to generate a plasmid wherein the BEV polymerase fragment from pCR.BEV.2 is inserted in the antisense orientation, relative to the CMV IE promoter sequence, using this cloning strategy. The present invention further encompasses such a genetic construct.

EXAMPLE 5

Synthetic genes and genetic constructs comprising the tyrosinase open reading frame

Isolation of the tyrosinase open reading frame

The tyrosinase structural gene is isolated by polymerase chain reaction, from mRNA derived from murine cells, using the following oligonucleotide primers under standard polymerase

Printed: 22-04-2005 TIPA1 99910592

chain reaction conditions:

Tyr 5'(forward primer; SEQ ID NO:9):

5'-CCCGGGGCTTAGTGTAAAACAGGCTGAGAG-3'; and

Tyr 3' (reverse primer; SEQ ID NO:10):

5'-CCCGGGCAAATCCCAGTCATTTCTTAGAAAC-3'.

Nucleotide residues 1 to 6 in each primer represent a *Sma*I cloning site. Nucleotides 7 to 30 of primer Tyr 5' correspond to the 5'-end of the murine tyrosinase cDNA sequence disclosed in GenBank Accession No. M20234 (Kwon *et al*, 1988). Nucleotides 7 to 31 of primer Tyr 3' correspond to the complement of the nucleotide sequence of the 3'-end of the murine tyrosinase cDNA sequence.

Plasmid pCR.tyr

Plasmid pCR.tyr is produced by sub-cloning the amplified tyrosinase structural gene into plasmid pCR2.1 (Example 1), substantially according to the manufacturer's protocol. Plasmid pCR.tyr can be used as a base plasmid to produce a range of genetic constructs designed to express the tyrosinase structural gene or a multiple structural gene unit comprising same, under the control of one or more promoter sequences.

Plasmid pCMV.TYR

Plasmid pCMV.TYR (Figure 23) comprises the complete mouse tyrosinase cDNA sequence placed operably in connection, in the sense orientation, with the CMV-IE promoter sequence and upstream of the SV40 polyadenylation sequence. To produce pCMV.TYR, the full-length mouse tyrosinase cDNA sequence was excised from plasmid pCR.tyr by digestion with *Sma*I and then ligated, into the *Sma*I cloning site of pCMV.cass (Figure 2). Clones possessing the tyrosinase structural gene in the sense orientation relative to the CMV-IE promoter were then selected.

45 20-04-2005

Plasmid pCMV.TYRLIB

Plasmid pCMV.TYRLIB (Figure 24) comprises a structural gene or multiple structural gene unit which comprises one or more tyrosinase gene fragments of 100 to 200 base pairs in length each, placed operably in connection with the CMV-IE promoter sequence and upstream of the SV40 polyadenylation signal. To produce pCMV.TYRLIB, blunt-ended fragments of the tyrosinase gene are ligated into *Sma*I-digested, dephosphorylated plasmid pCMV.cass DNA (Figure 2). The tyrosinase gene fragments are produced, for example, by sonication or mechanical shearing and end-repair using T4 DNA polymerase. Accordingly, the structural gene insert in plasmid pCMV.TYRLIB is variable and an representative library of pCMV.TYRLIB plasmids, covering the complete tyrosinase gene sequence, may be produced using such procedures. The present invention clearly encompasses such representative libraries. Those skilled in the art will recognise that such procedures are also useful for structural genes other than tyrosinase and, as a consequence, the present invention clearly extends to synthetic genes and genetic constructs wherein the structural gene present in pCMV.TYRLIB is a structural gene other than a tyrosinase gene fragment.

EXAMPLE 6

Synthetic genes and genetic constructs comprising the *lac*I open reading frame

Plasmid pCMV.Lac

Plasmid pCMV.Lac (Figure 25) contains a CMV IE promoter driving expression of the lac repressor protein encoded by the *Escherichia coli lac*I gene. Accordingly, the open reading frame of the *Lac*I gene is cloned in the sense orientation with respect to the CMV IE promoter sequence in this plasmid. This construct also contains the selectable marker for neomycin resistance.

To produce plasmid pCMV.Lac, the *lac*I gene was excised from plasmid pCMV.LacI (Stratagene) by digestion with *Hin*dIII and *Bsa*BI and then ligated, in the sense orientation, into the multiple cloning site (MCS) of plasmid pCMV.cass (Figure 2) which had been digested with *Hin*dIII and *Sma*I.

Plasmid pCMVLacI.OPRSV1.cass

Plasmid pCMVLacI.OPRSV1.cass (Figure 26) is a dual expression construct in which the CMV-IE promoter drives expression of the *Lac*I structural gene to produce the lac repressor protein and the OPRSVI promoter drives the expression of a second structural gene or multiple structural gene unit placed operably under control of lac repressor protein.

To produce plasmid pCMVLac.OPRSV1.cass, a DNA fragment comprising the OPRSVI promoter, SV40 intron, *lac* operator sequence, multiple cloning site (MCS) and TK poly(A) sequence was excised from plasmid pOPRSVI/MCS (Stratagene), by digestion with *Sna*B1 and *Ase*1 restriction enzymes , then end-filled using *Pfu*I polymerase and ligated into the end-filled *Bgl*II cloning site of plasmid pCMVLacI (Stratagene).

EXAMPLE 7

Synthetic genes and genetic constructs comprising the *lac*I and green fluorescent protein (GFP)open reading frames

Plasmid pCMVLacI.OPRSV1.GFP.cass

Plasmid pCMVLacI.OPRSVI.GFP.cass (Figure 27) is designed such that a structural gene or multiple structural gene unit can be fused to the 3 ' untranslated region of the *lac*I gene, by cloning directly into the unique *Bsa*B1 cloning site which is located after the *lac*I stop codon and before an SV40 polyadenylation signal. Alternatively, the *BSA*B1 site may be modified to facilitate cloning, for example by the addition of linkers or adaptors. This construct also contains the antibiotic selectable marker for hygromycin resistance.

To produce plasmid pCMVLacI.OPRSVI.GFP.cass, the enhanced GFP coding sequence was excised from plasmid pEGFP-N1 MCS (Figure 1) by digestion with *Xho*I and *Not*I and the DNA fragment thus produced was ligated into the *Xho*I and *Not*I cloning sites of the multiple cloning site present in plasmid pCMVLacI.OPRSVI.cass (Figure 26).

EXAMPLE 8

Synthetic genes and genetic constructs comprising the *lac*I and green fluorescent protein (GFP) and tyrosinase open reading frames

Plasmid pCMVLacI.TYR.OPRSV1.GFP

Plasmid pCMVLacI.TYR.OPRSV1.GFP (Figure 28) is a dual construct in which the CMV IE promoter drives expression of the *lac*I gene and the mRNA of the mouse tyrosinase cDNA or a fragment thereof, whilst the OPRSVI promoter drives expression of GFP operably under control of the *lac*I gene. The construct is designed such that the mouse tyrosinase gene is fused to the 3 ' untranslated region of the *lac*I gene via a unique *Bsa*B1 cloning site. This cloning site is located after the stop codon of the *lac*I coding sequence, but before the SV40 polyadenylation signal. The construct also contains the hygromycin-resistance gene as a selection marker.

To produce plasmid pCMVLacI.TYR.OPRSV1.GFP, the complete tyrosinase gene present in plasmid pCR.tyr (Stratagene; Example 1) is isolated from host cells, digested with *Sma*I and ligated into *Bsa*B1-digested and dephosphorylated plasmid pCMVLacI.OPRSVI.GFP.cass DNA (Figure 27).

Printed: 22-04-2005 TIPA1 99910592

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: AgGene Australia Pty.Ltd and The Government of Queensland as represented by Queensland Department of Primary Industries

ii) TITLE OF INVENTION: Synthetic genes and genetic constructs comprising same I

iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: DAVIES COLLISON CAVE

(B) STREET: 1 LITTLE COLLINS STREET

(C) CITY: MELBOURNE

(D) STATE: VICTORIA

(E) COUNTRY: AUSTRALIA

(F) ZIP: 3000

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:AU provisional

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: HUGHES EL, JOHN E L

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: +61 3 9254 2777

(B) TELEFAX: +61 3 9254 2770

(C) TELEX: AA 31787

949 20-04-2005

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGCAGATCT AACAATGGCA GGACAAATCG AGTACATC 38

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCGGGATCC TCGAAAGAAT CGTACCACTT C 31

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGCGGATCC TTAGAAAGAA TCGTACCAC 29

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGCAGATCT GGACAAATCG AGTACATC 28

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGATCTGTAA ACGGCCACAA GTTCAG 26

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGATCCTTGT ACAGCTCGTC CATGCC 26

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTCGACAATA AAATATCTTT ATTTTCATTA CATCTGTGTG TTGGTTTTTT GTGTGATTTT 60

TGCAAAAGCC TAGG 74

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCGACGTTT AGAGCAGAAG TAACACTTCC G 31

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCGGGGCTT AGTGTAAAAC AGGCTGAGAG 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCCGGGCAAA TCCCAGTCAT TTCTTAGAAA C 31

22-04-2005 TIPAT 99910592

EQUIVALENTS

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

REFERENCES

1. Ausubel, F.M. *et al.*(1987) *In:* Current Protocols in Molecular Biology, Wiley Interscience (ISBN 047140338).

2. Chalfie,M. *et al* (1994) *Science* 263: 802-805.

3. Cormack, B. *et al* (1996) *Gene* 173: 33-38.

4. Inouye, S. and Tsuji, F.I. (1994) *FEBS Letts.* 341: 277-280.

5. Kwon, B.S. *et al.* (1988) *Biochem. Biophys. Res. Comm.* 153:1301- 1309.

6. Prasher, D.C. *et al.* (1992) *Gene*111: 229-233.

DATED this 19TH day of MARCH, 1998

Ag-Gene Australia Ltd AND

State of Queensland through its Department of Primary Industries

by DAVIES COLLISON CAVE

Patent Attorneys for the Applicants

20-04-2005

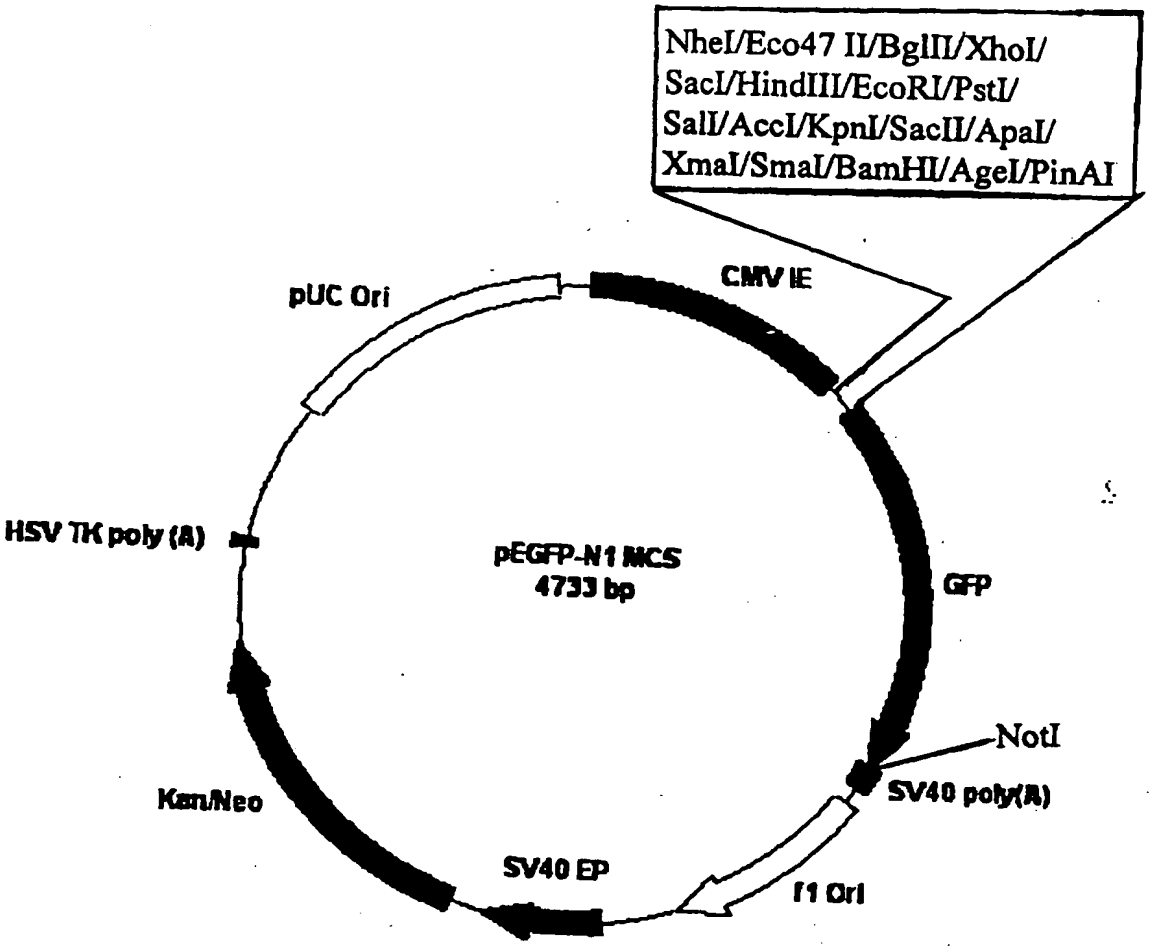

NheI/Eco47 II/BglII/XhoI/
SacI/HindIII/EcoRI/PstI/
SalI/AccI/KpnI/SacII/ApaI/
XmaI/SmaI/BamHI/AgeI/PinAI
CMV IE
pUC Ori
HSV TK poly (A)
pEGFP-N1 MCS
4733 bp
GFP
NotI
SV40 poly(A)
Kan/Neo
SV40 EP
f1 Ori

FIGURE 1

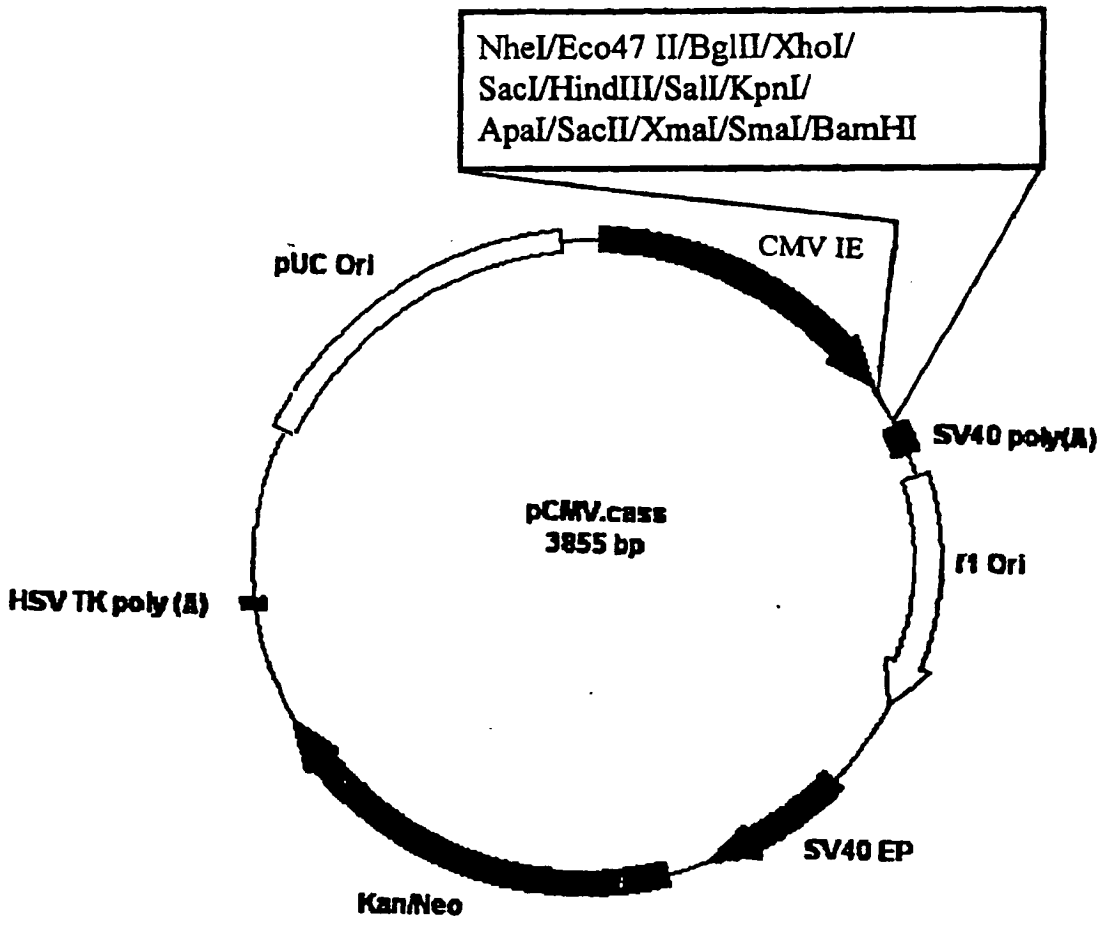

NheI/Eco47 II/BglII/XhoI/
SacI/HindIII/SalI/KpnI/
ApaI/SacII/XmaI/SmaI/BamHI
CMV IE
pUC Ori
SV40 poly(A)
pCMV.cass
3855 bp
f1 Ori
HSV TK poly (A)
SV40 EP
Kan/Neo

FIGURE 2

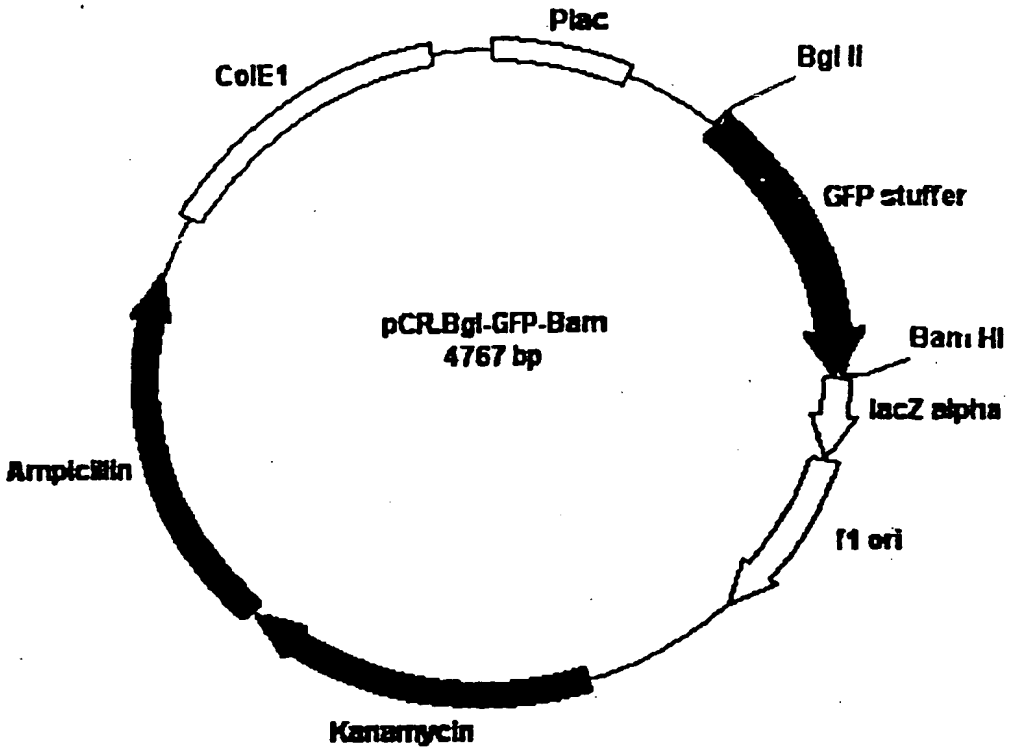

Plac
Bgl II
ColE1
GFP stuffer
pCRLBgl-GFP-Bam
4767 bp
Bam HI
lacZ alpha
Ampicillin
f1 ori
Kanamycin

FIGURE 3

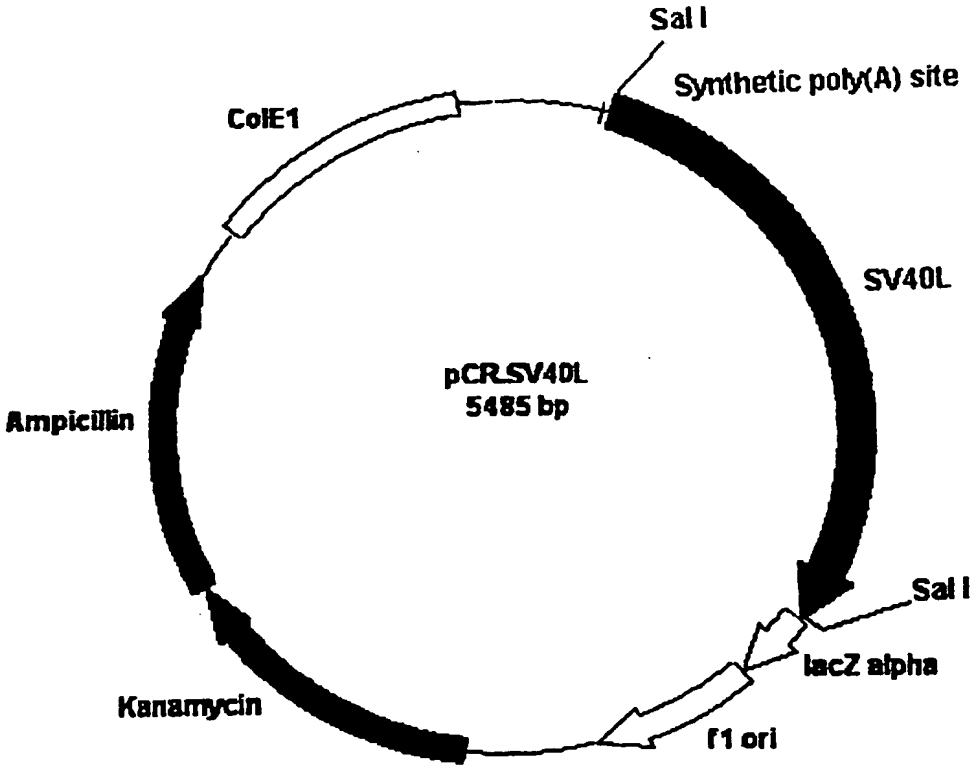

Sal I
Synthetic poly(A) site
ColE1
SV40L
pCR.SV40L
5485 bp
Ampicillin
Sal I
lacZ alpha
Kanamycin
f1 ori

FIGURE 4

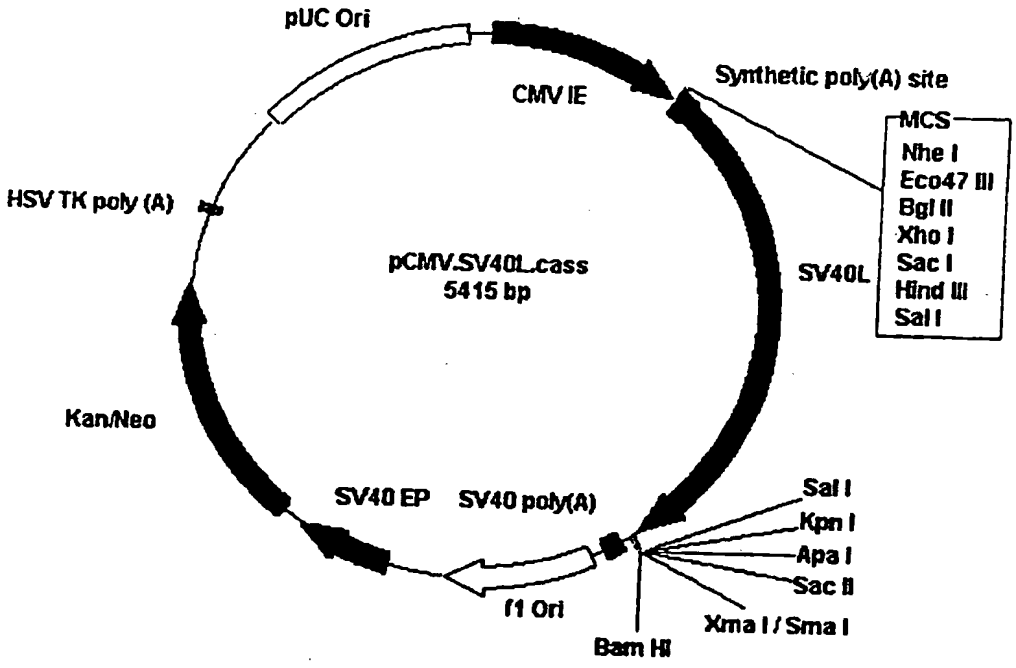

pUC Ori
CMV IE
Synthetic poly(A) site
MCS
Nhe I
Eco47 III
Bgl II
Xho I
Sac I
Hind III
Sal I
SV40L
HSV TK poly (A)
pCMV.SV40L.cass
5415 bp
Kan/Neo
SV40 EP
SV40 poly(A)
Sal I
Kpn I
Apa I
Sac II
Xma I / Sma I
f1 Ori
Bam HI

FIGURE 5

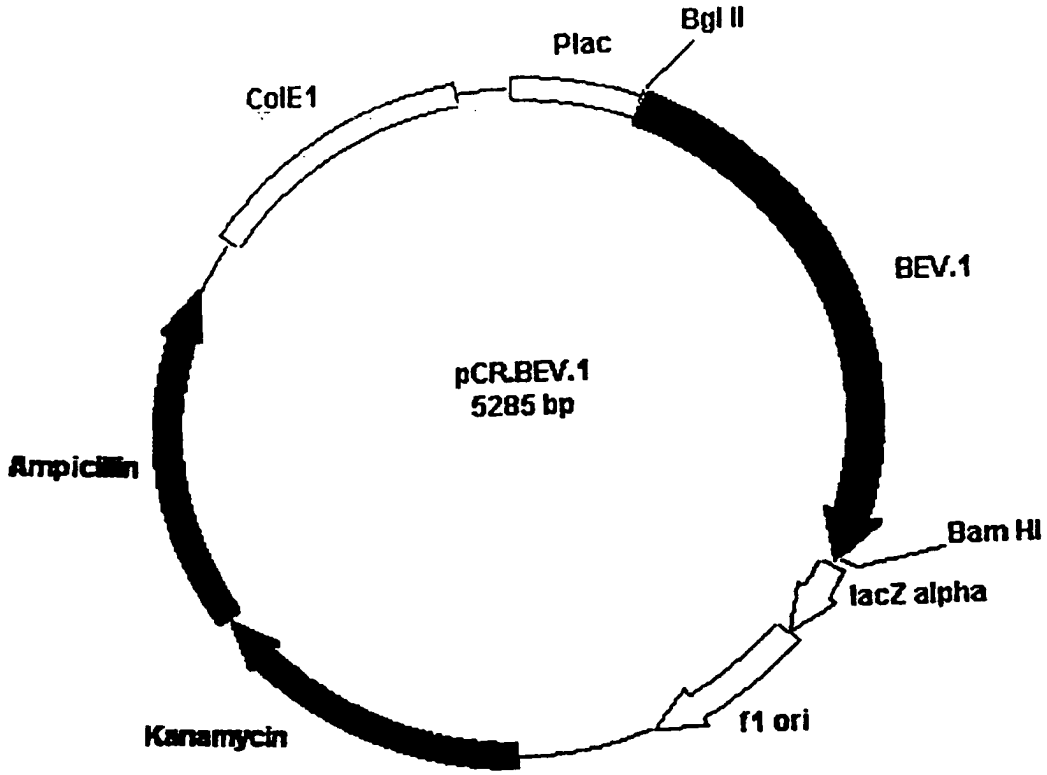

Bgl II
Plac
ColE1
BEV.1
pCR.BEV.1
5285 bp
Ampicillin
Bam HI
lacZ alpha
f1 ori
Kanamycin

FIGURE 6

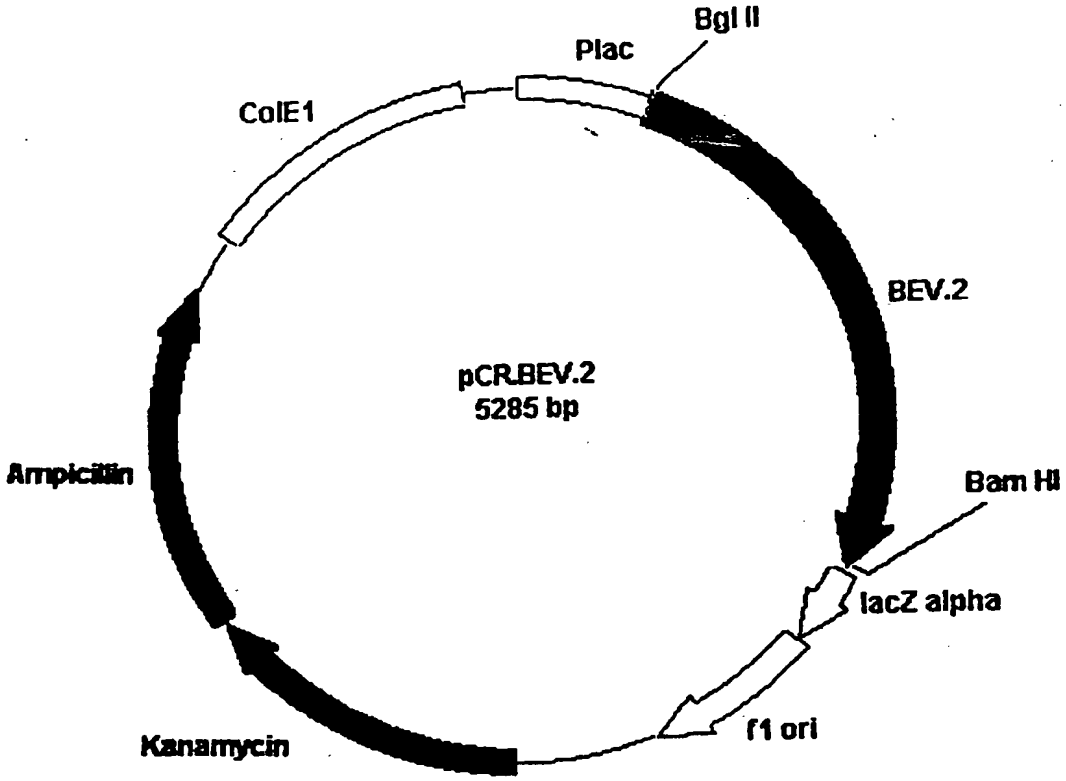

Bgl II
Plac
ColE1
BEV.2
pCR.BEV.2
5285 bp
Ampicillin
Bam HI
lacZ alpha
f1 ori
Kanamycin

FIGURE 7

8/28

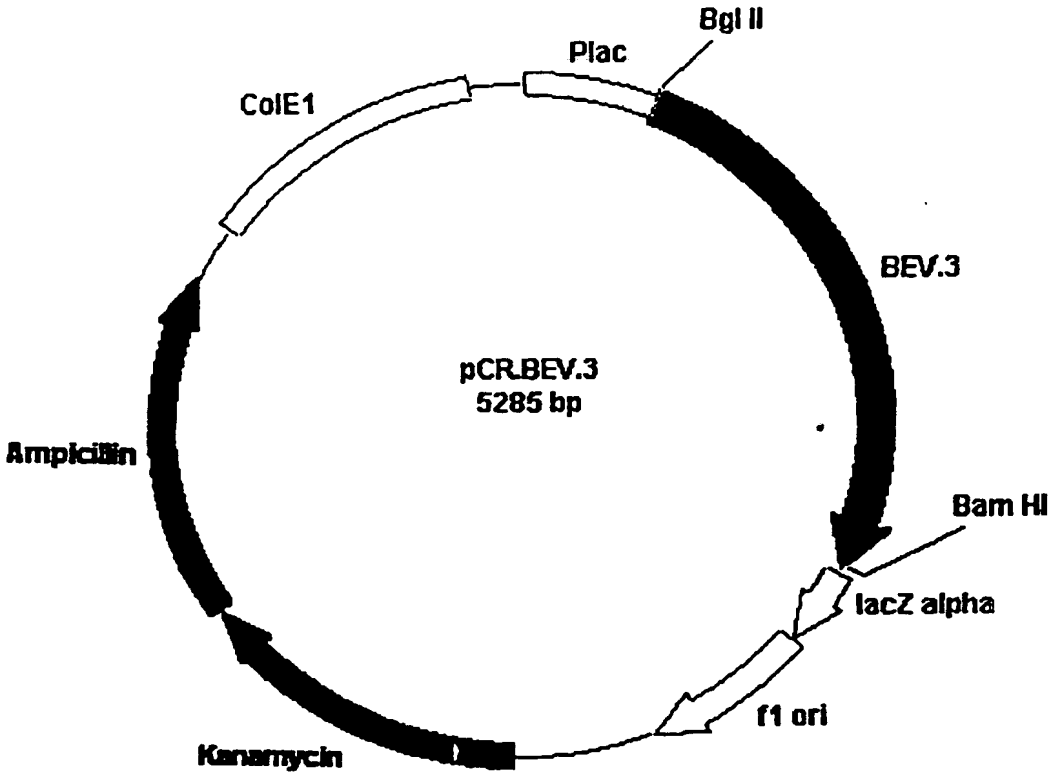

Bgl II
Plac
ColE1
BEV.3
pCR.BEV.3
5285 bp
Ampicillin
Bam HI
lacZ alpha
f1 ori
Kanamycin

FIGURE 8

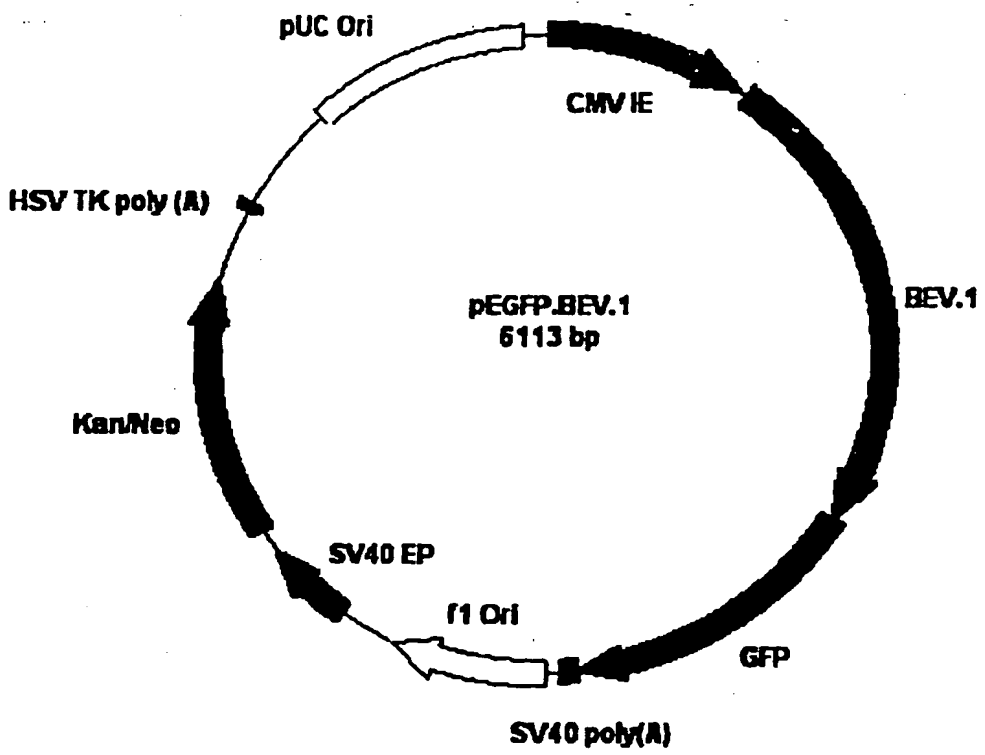

pUC Ori
CMV IE
HSV TK poly (A)
pEGFP.BEV.1
6113 bp
BEV.1
Kan/Neo
SV40 EP
f1 Ori
GFP
SV40 poly(A)

FIGURE 9

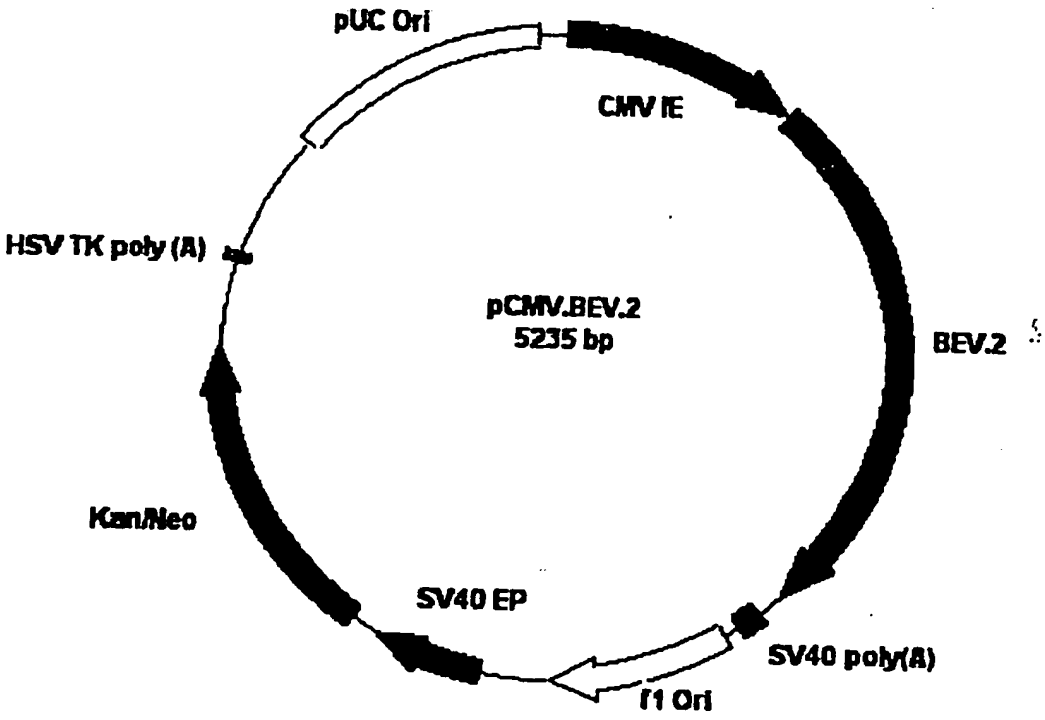

pUC Ori
CMV IE
HSV TK poly (A)
pCMV.BEV.2
5235 bp
BEV.2
Kan/Neo
SV40 EP
SV40 poly(A)
f1 Ori

FIGURE 10

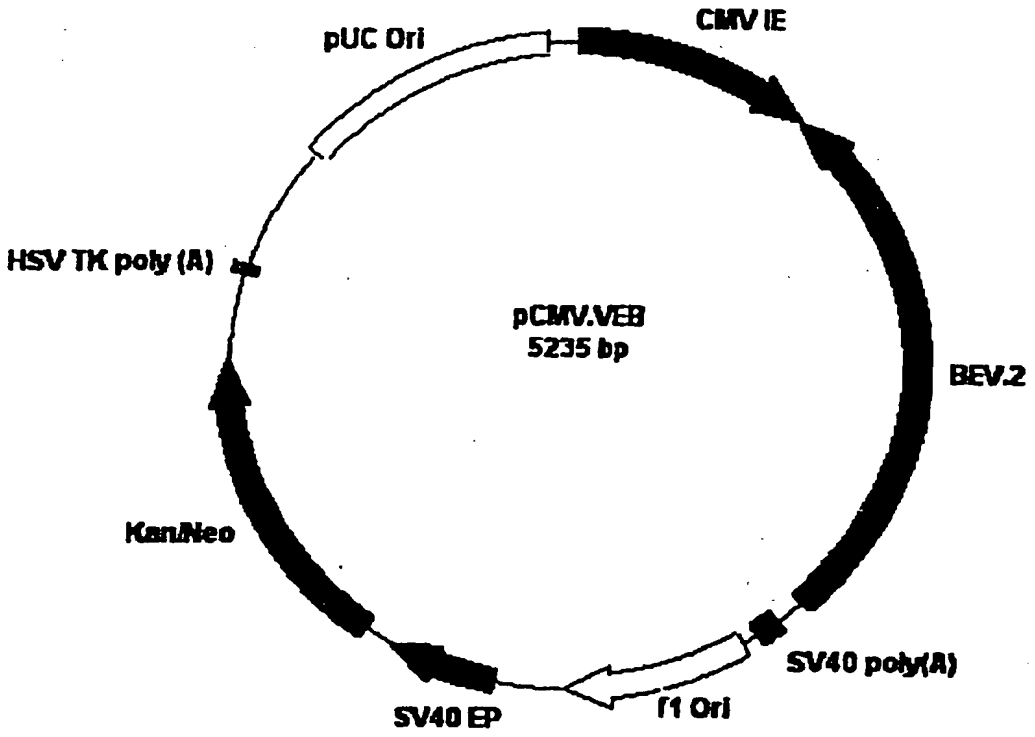

pUC Ori
CMV IE
HSV TK poly (A)
pCMV.VEB
5235 bp
BEV.2
Kan/Neo
SV40 poly(A)
SV40 EP
f1 Ori

FIGURE 11

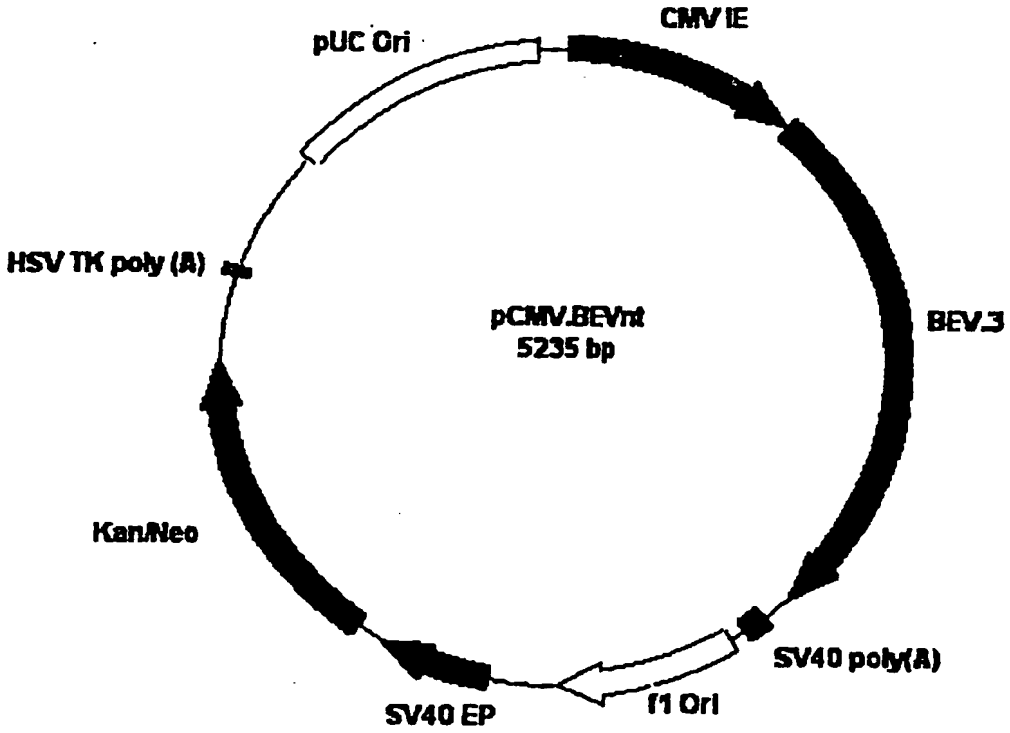

pUC Ori
CMV IE
HSV TK poly (A)
pCMV.BEVnt
5235 bp
BEV.3
KanNeo
SV40 poly(A)
SV40 EP
f1 Ori

FIGURE 12

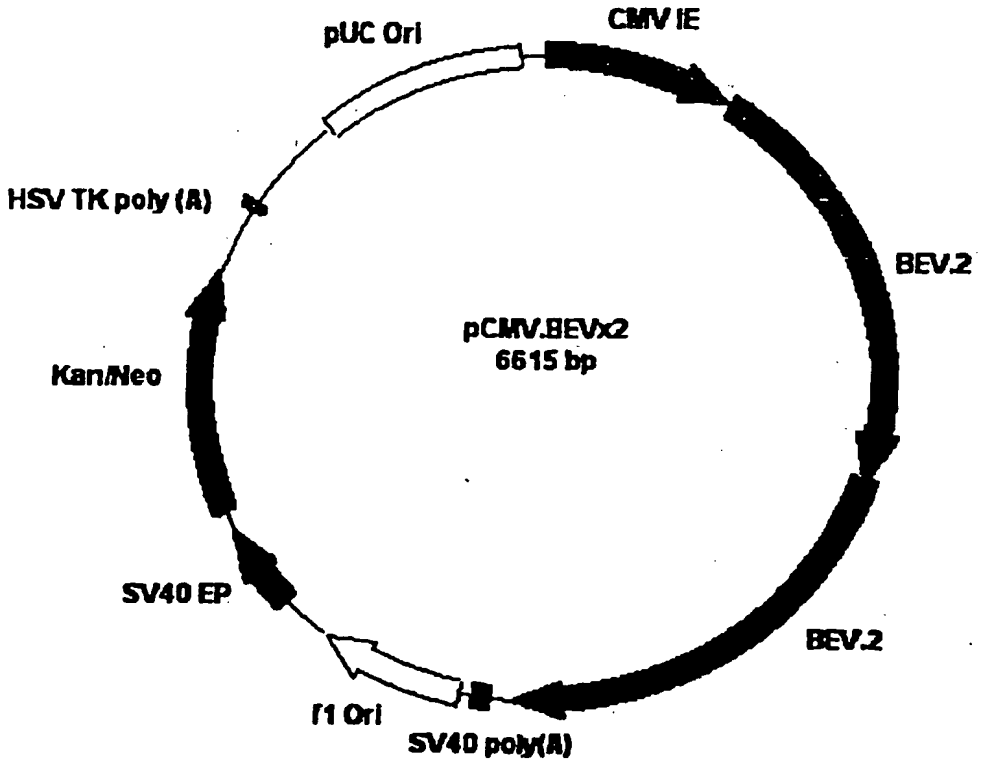

pUC Ori
CMV IE
HSV TK poly (A)
BEV.2
pCMV.BEVx2
6615 bp
Kan/Neo
SV40 EP
BEV.2
f1 Ori
SV40 poly(A)

FIGURE 13

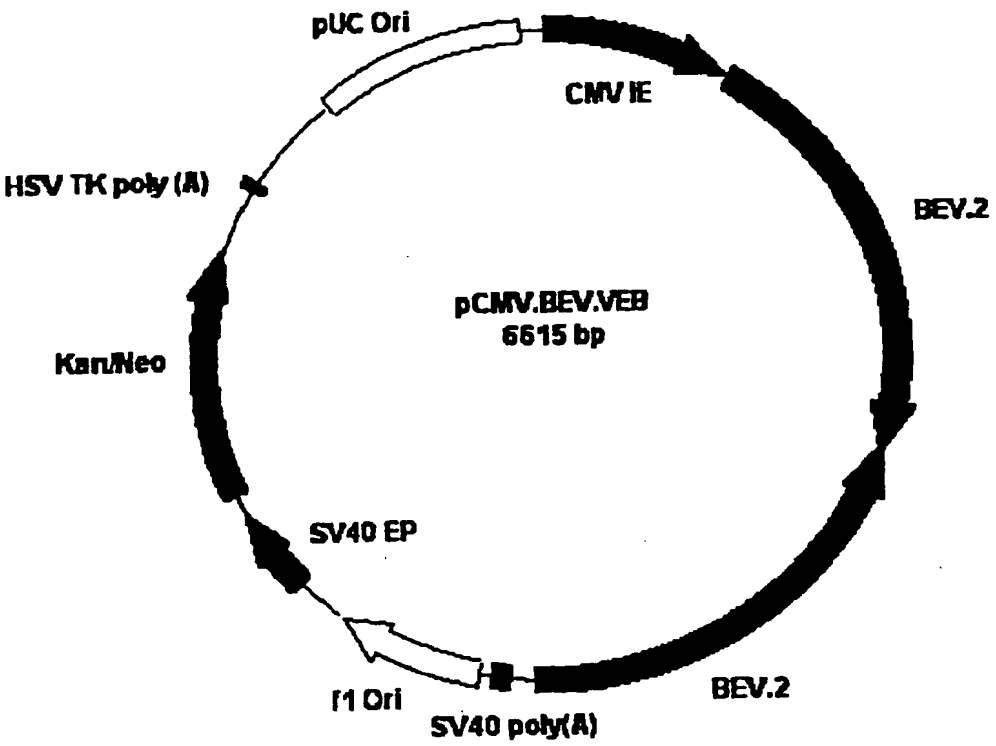

pUC Ori
CMV IE
HSV TK poly (A)
BEV.2
pCMV.BEV.VEB
6615 bp
KanrNeo
SV40 EP
f1 Ori
BEV.2
SV40 poly(A)

FIGURE 14

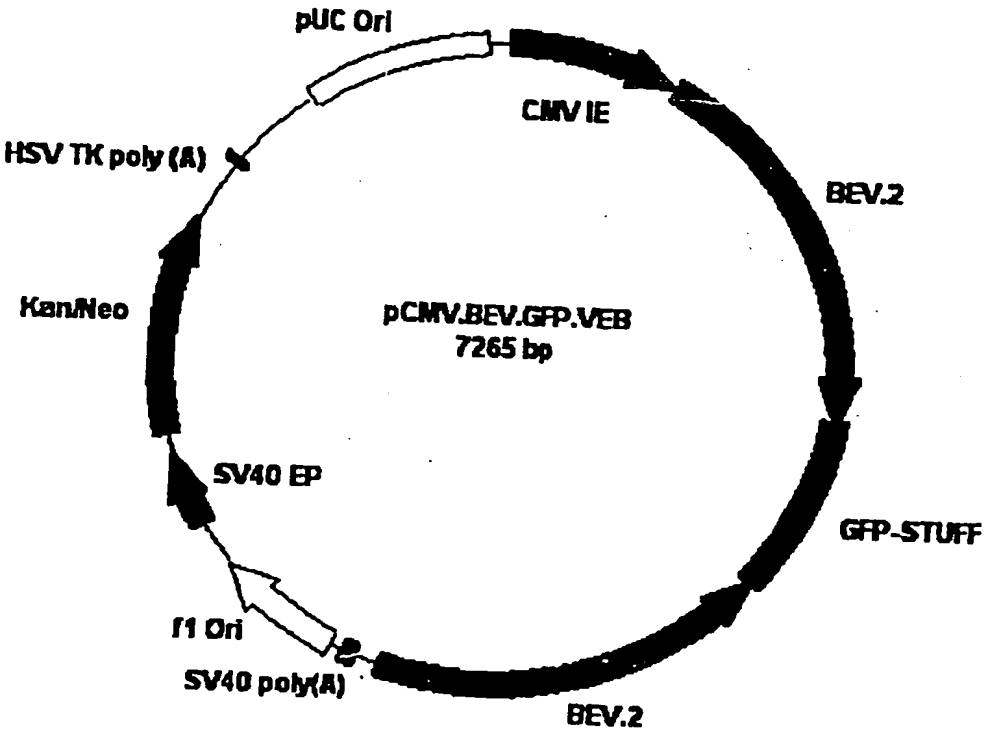

pUC Ori
CMV IE
BEV.2
GFP-STUFF
BEV.2
SV40 poly(A)
f1 Ori
SV40 EP
KanNeo
HSV TK poly (A)
pCMV.BEV.GFP.VEB
7265 bp

FIGURE 15

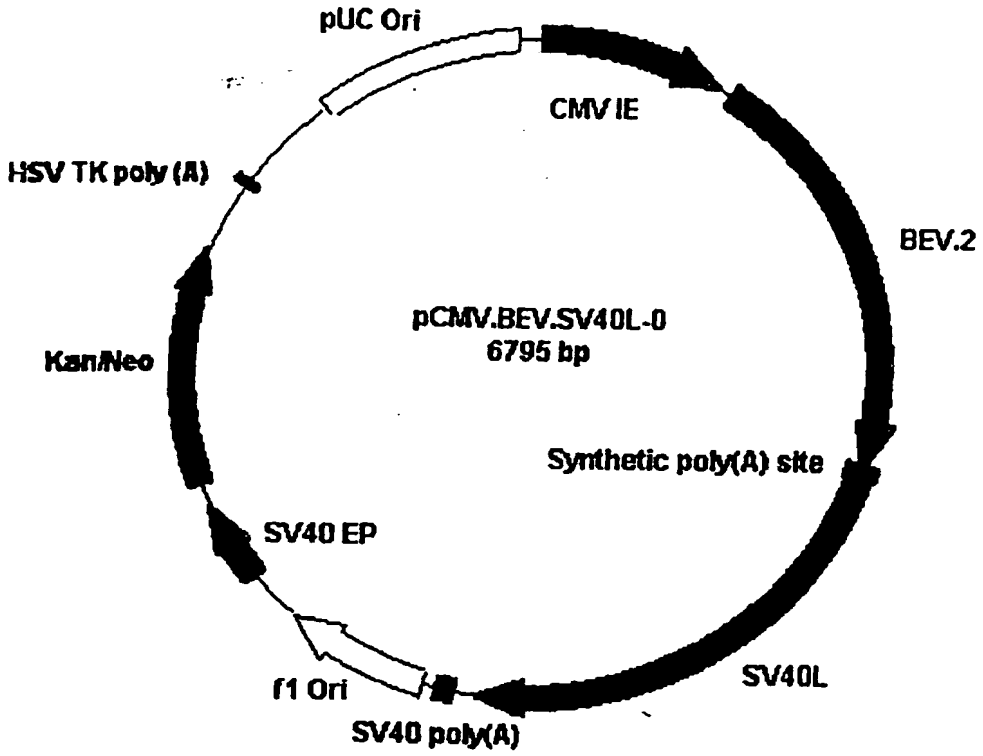

pUC Ori
CMV IE
HSV TK poly (A)
BEV.2
pCMV.BEV.SV40L-0
6795 bp
KanNeo
Synthetic poly(A) site
SV40 EP
f1 Ori
SV40L
SV40 poly(A)

FIGURE 16

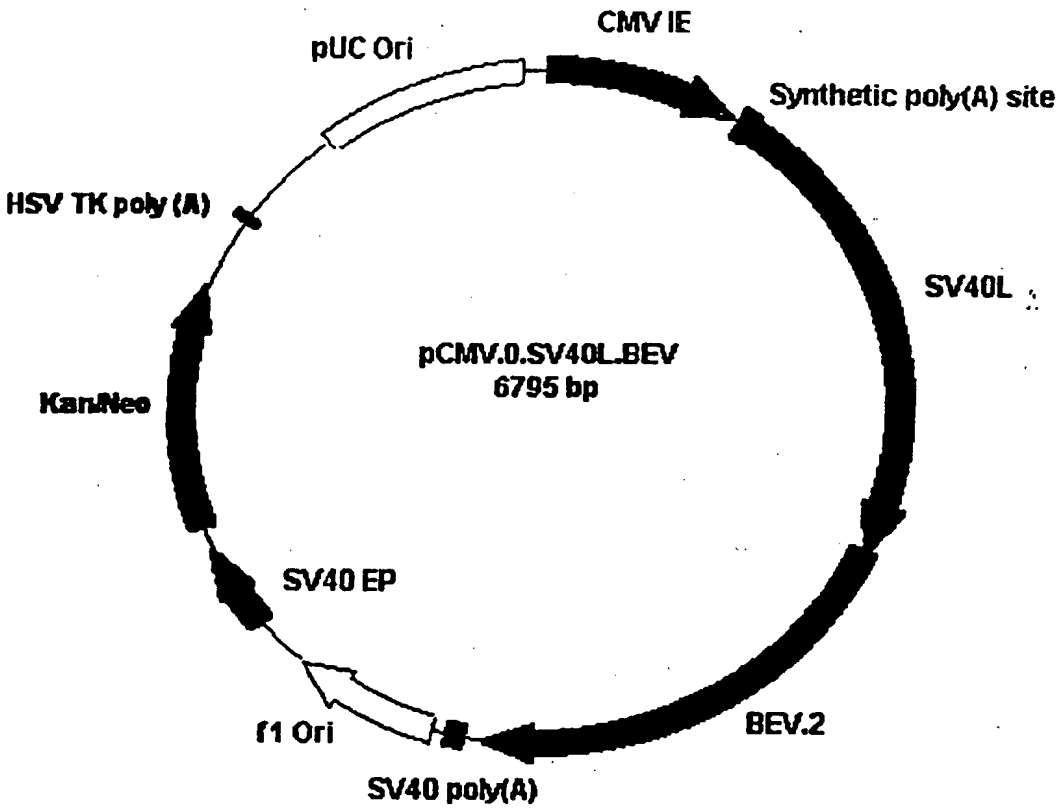

CMV IE
pUC Ori
Synthetic poly(A) site
HSV TK poly (A)
SV40L
pCMV.0.SV40L.BEV
6795 bp
KanNeo
SV40 EP
f1 Ori
BEV.2
SV40 poly(A)

FIGURE 17

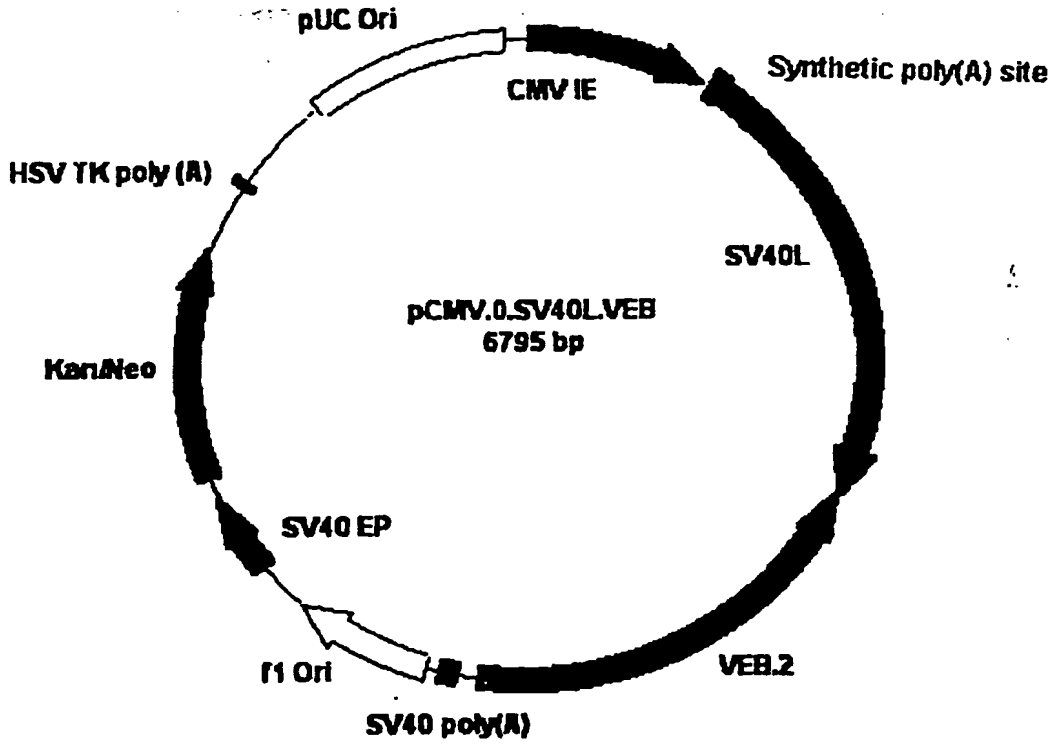

pUC Ori
CMV IE
Synthetic poly(A) site
HSV TK poly (A)
SV40L
pCMV.0.SV40L.VEB
6795 bp
KanNeo
SV40 EP
f1 Ori
VEB.2
SV40 poly(A)

FIGURE 18

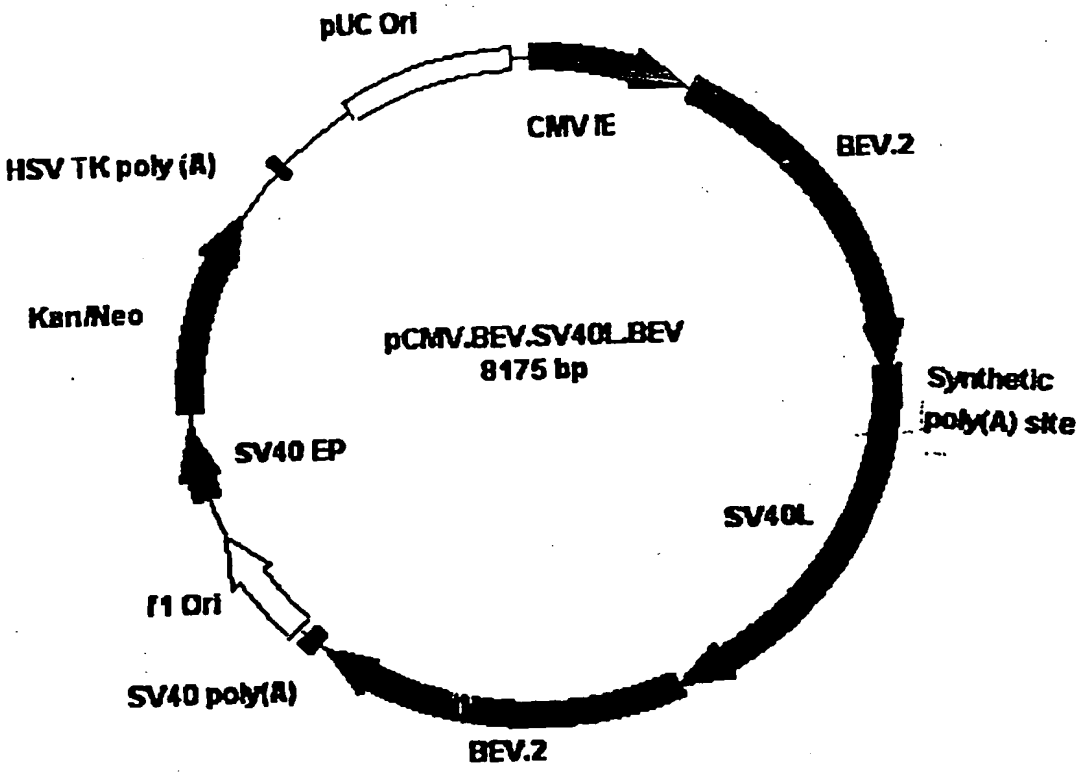

pUC Ori
CMV IE
BEV.2
HSV TK poly (A)
Kan/Neo
pCMV.BEV.SV40L.BEV
8175 bp
Synthetic
poly(A) site
SV40 EP
SV40L
f1 Ori
SV40 poly(A)
BEV.2

FIGURE 19

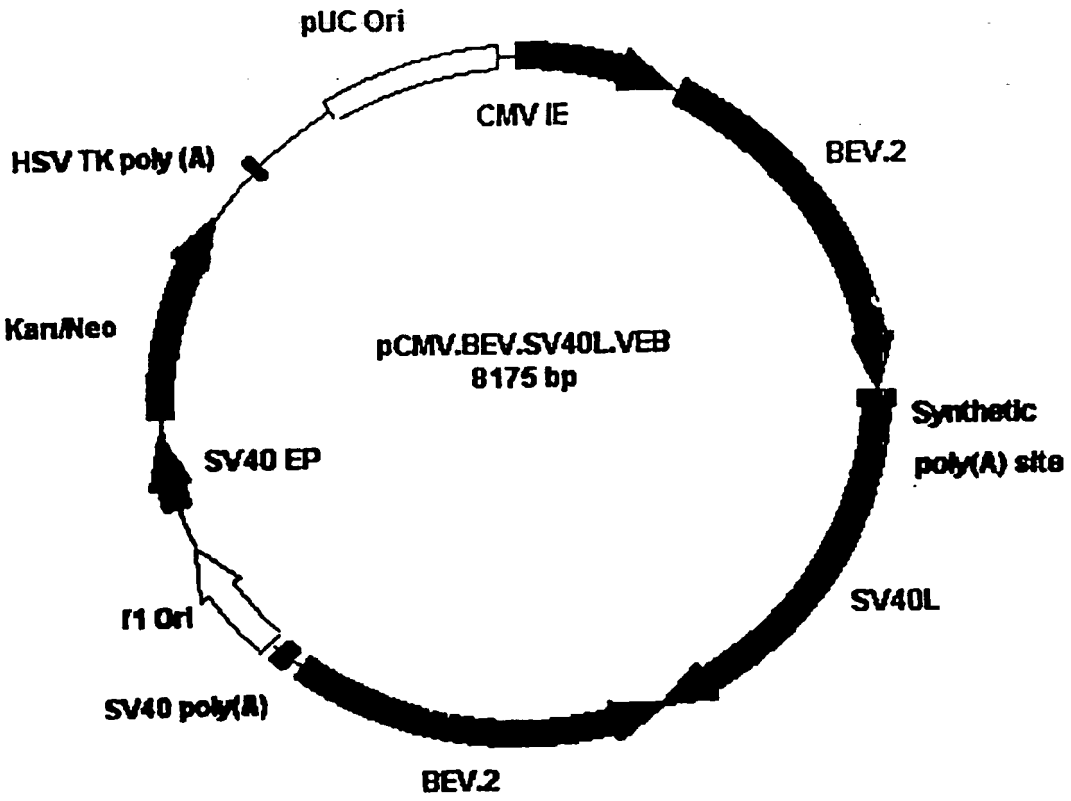

pUC Ori
CMV IE
BEV.2
HSV TK poly (A)
Kan/Neo
pCMV.BEV.SV40L.VEB
8175 bp
Synthetic
poly(A) site
SV40 EP
SV40L
f1 Ori
SV40 poly(A)
BEV.2

FIGURE 20

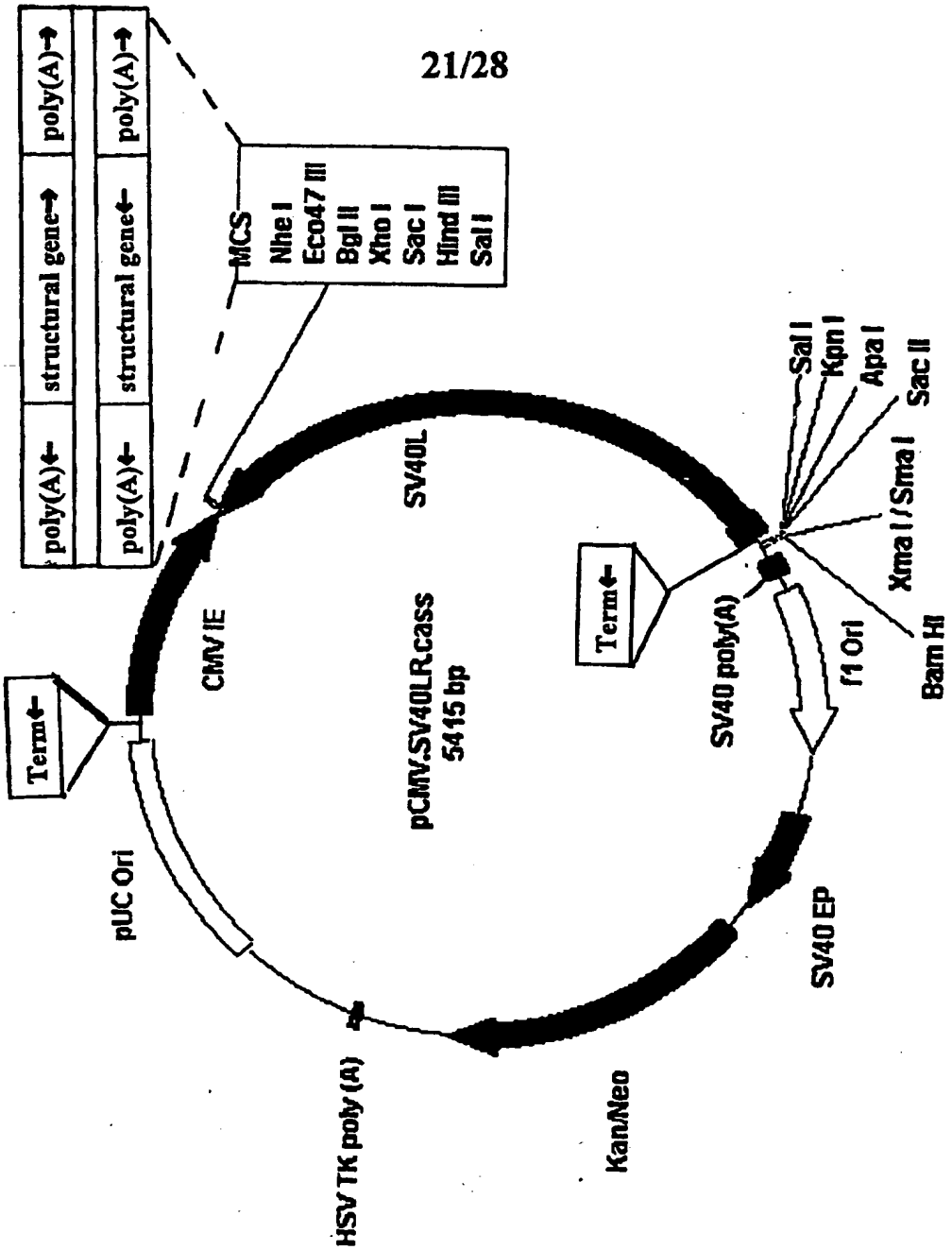

poly(A)
structural gene
poly(A)
poly(A)
structural gene
poly(A)
MCS
Nhe I
Eco47 III
Bgl II
Xho I
Sac I
Hind III
Sal I
SV40L
Sal I
Kpn I
Apa I
Sac II
Xma I / Sma I
Bam HI
f1 Ori
SV40 poly(A)
Term
Term
CMV IE
pCMV.SV40LR.cass
5415 bp
pUC Ori
HSV TK poly (A)
KanNeo
SV40 EP

FIGURE 21

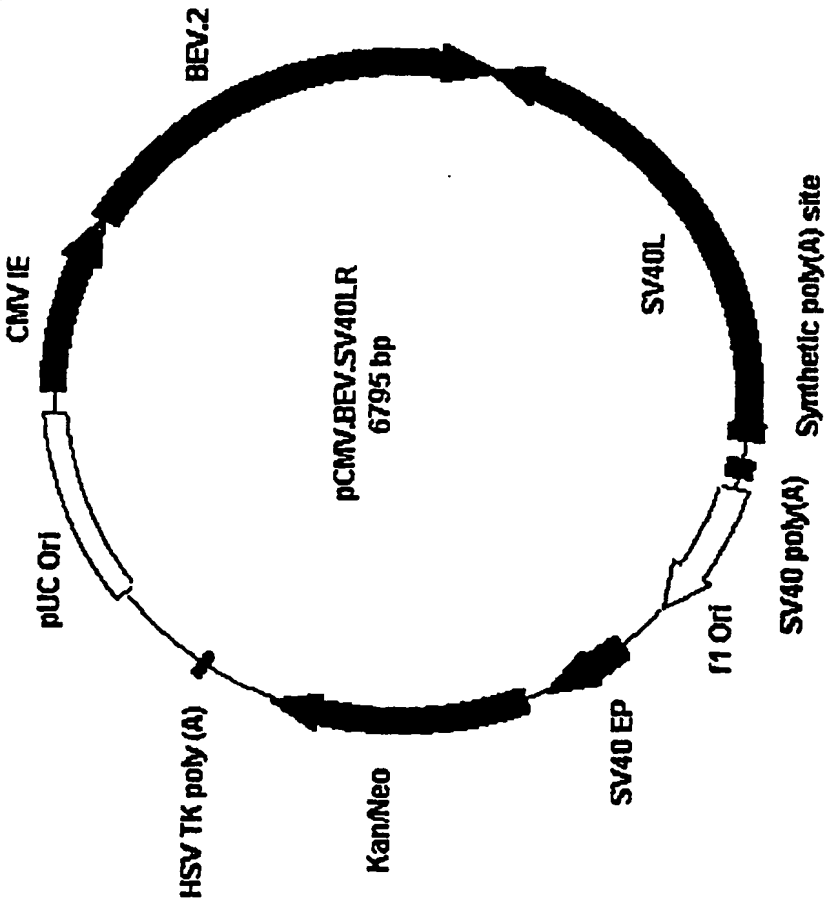

BEV.2
CMV IE
pCMV.BEV.SV40LR
6795 bp
SV40L
Synthetic poly(A) site
pUC Ori
SV40 poly(A)
f1 Ori
HSV TK poly (A)
Kan/Neo
SV40 EP

FIGURE 22

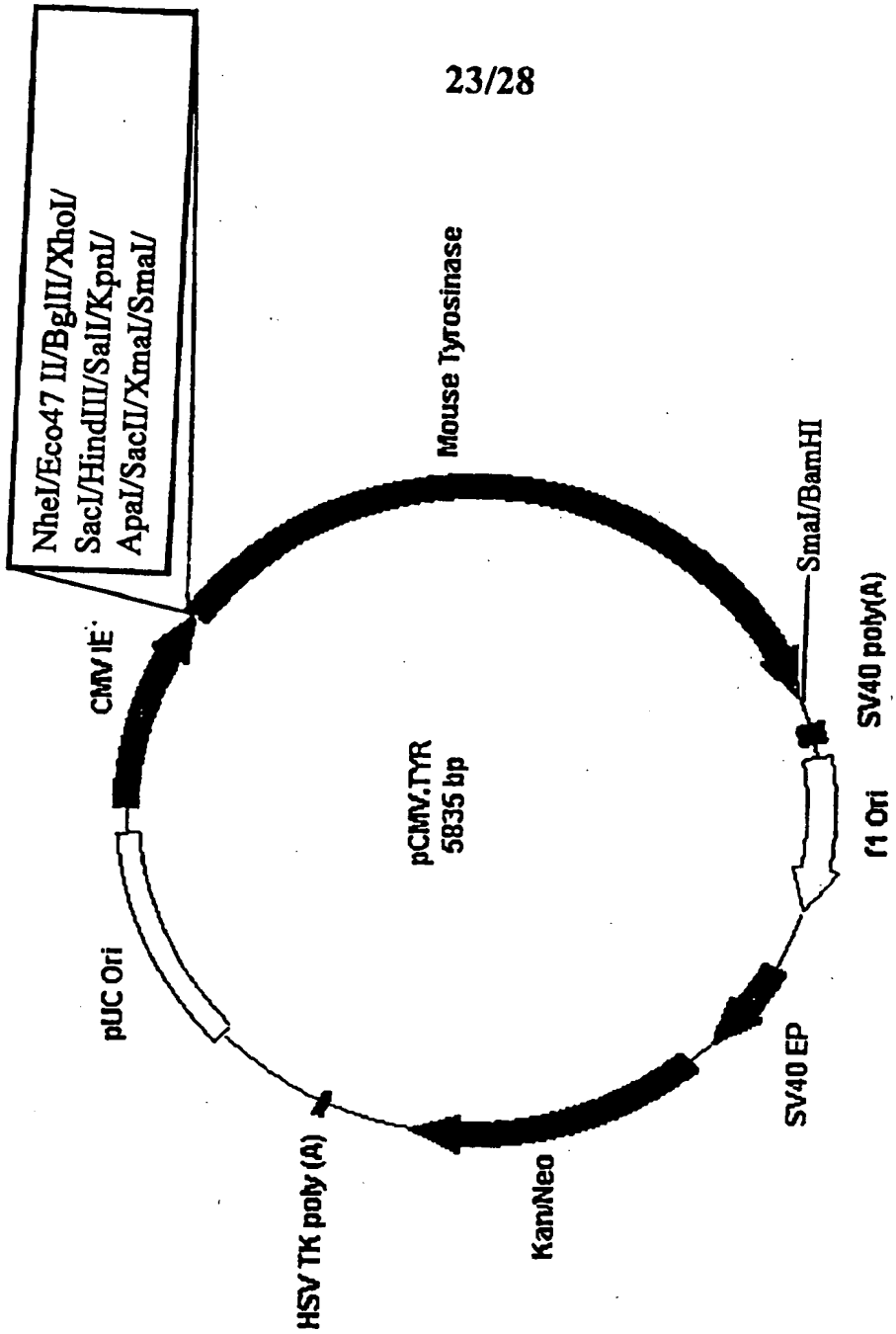

NheI/Eco47 II/BglII/XhoI/
SacI/HindIII/SalI/KpnI/
ApaI/SacII/XmaI/SmaI/
Mouse Tyrosinase
SmaI/BamHI
SV40 poly(A)
f1 Ori
SV40 EP
KanNeo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.TYR
5835 bp

FIGURE 23

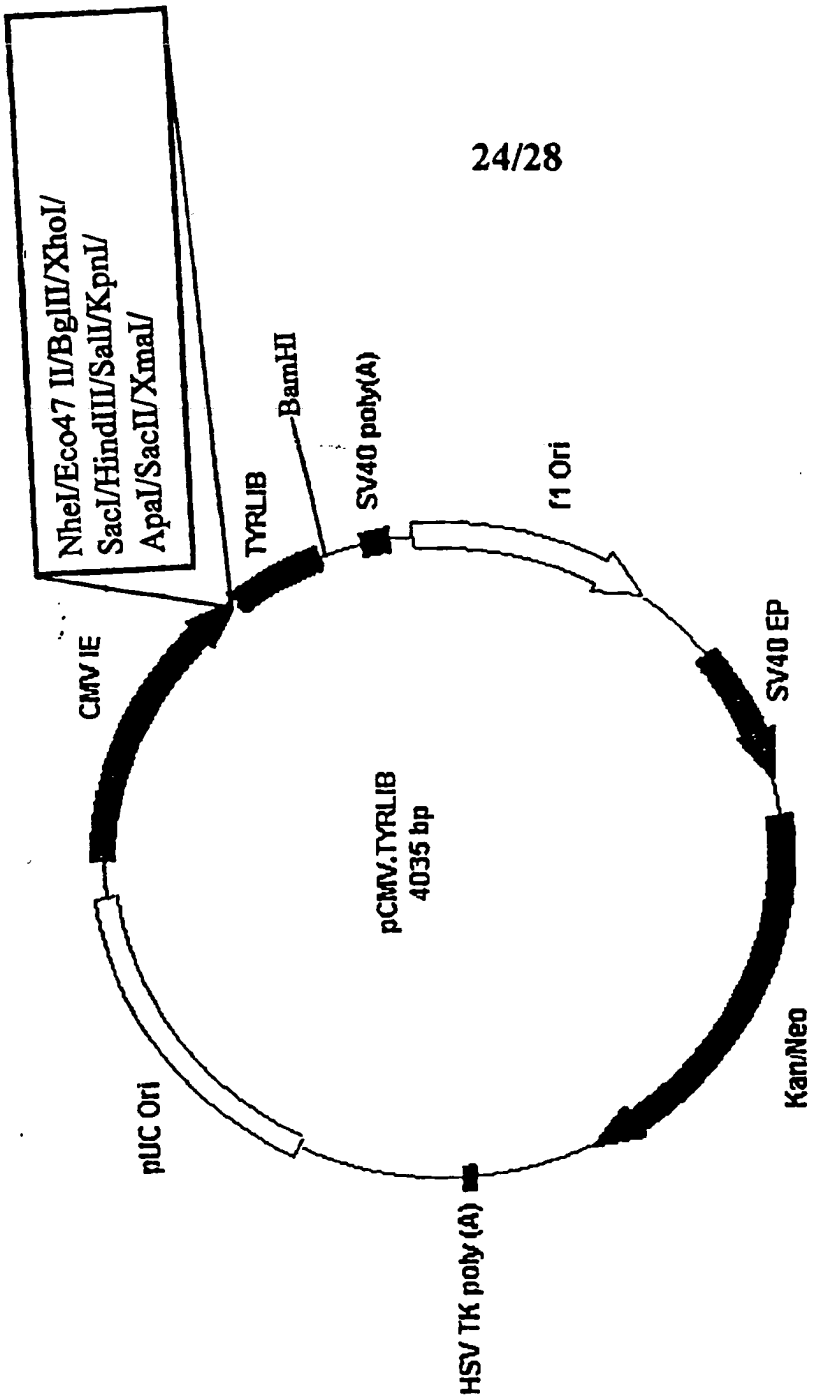

NheI/Eco47 II/BglII/XhoI/
SacI/HindIII/SalI/KpnI/
ApaI/SacII/XmaI/
TYRLIB
BamHI
SV40 poly(A)
f1 Ori
SV40 EP
Kan/Neo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.TYRLIB
4035 bp

FIGURE 24

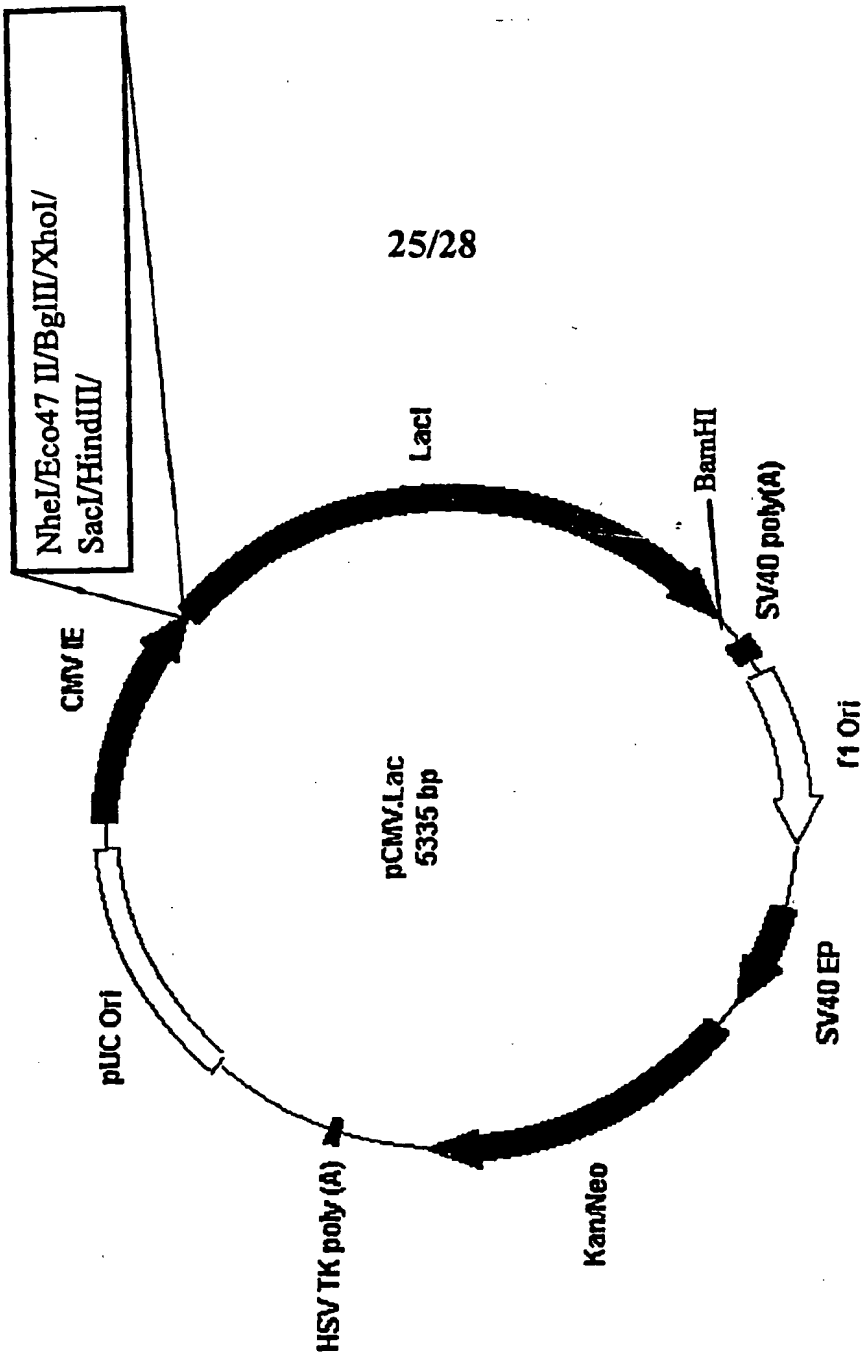

NheI/Eco47 II/BglII/XhoI/
SacI/HindIII/
LacI
BamHI
SV40 poly(A)
f1 Ori
SV40 EP
KanNeo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.Lac
5335 bp

FIGURE 25

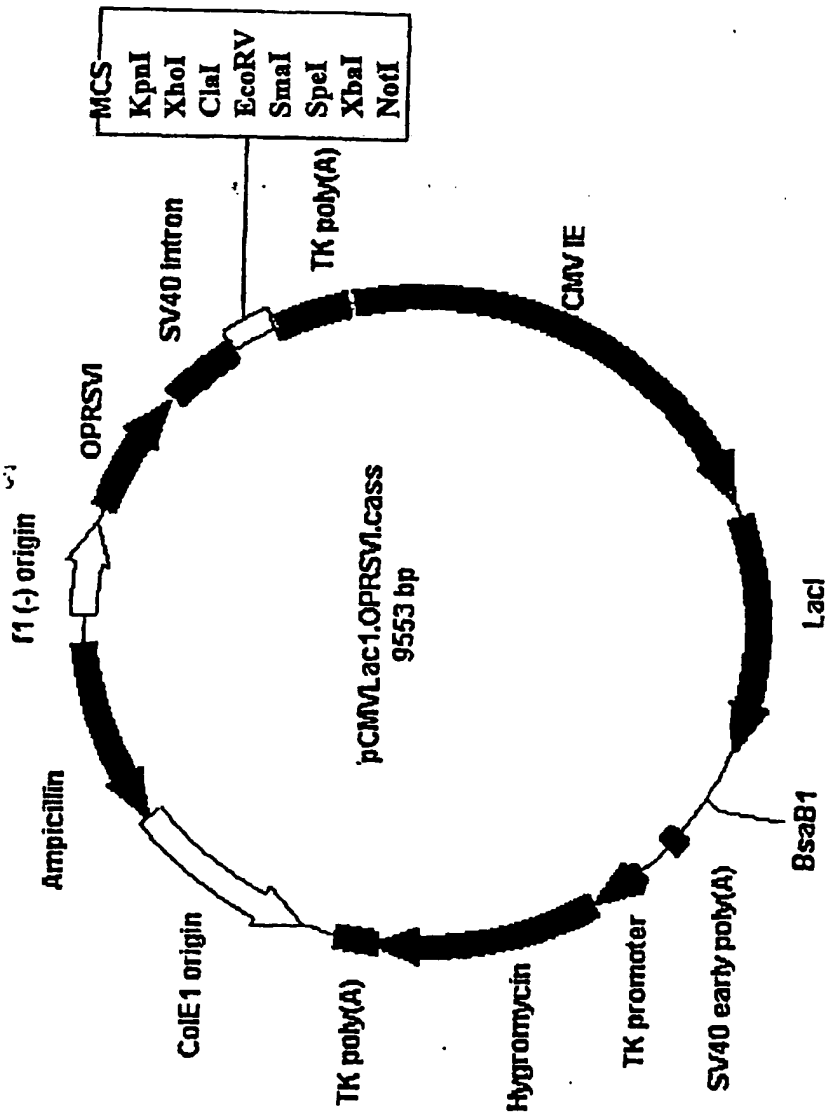

MCS
KpnI
XhoI
ClaI
EcoRV
SmaI
SpeI
XbaI
NotI
TK poly(A)
CMV IE
SV40 intron
OPRSVI
f1 (-) origin
pCMVLac1.OPRSVI.cass
9553 bp
LacI
Ampicillin
BsaB1
ColE1 origin
SV40 early poly(A)
TK poly(A)
TK promoter
Hygromycin

FIGURE 26

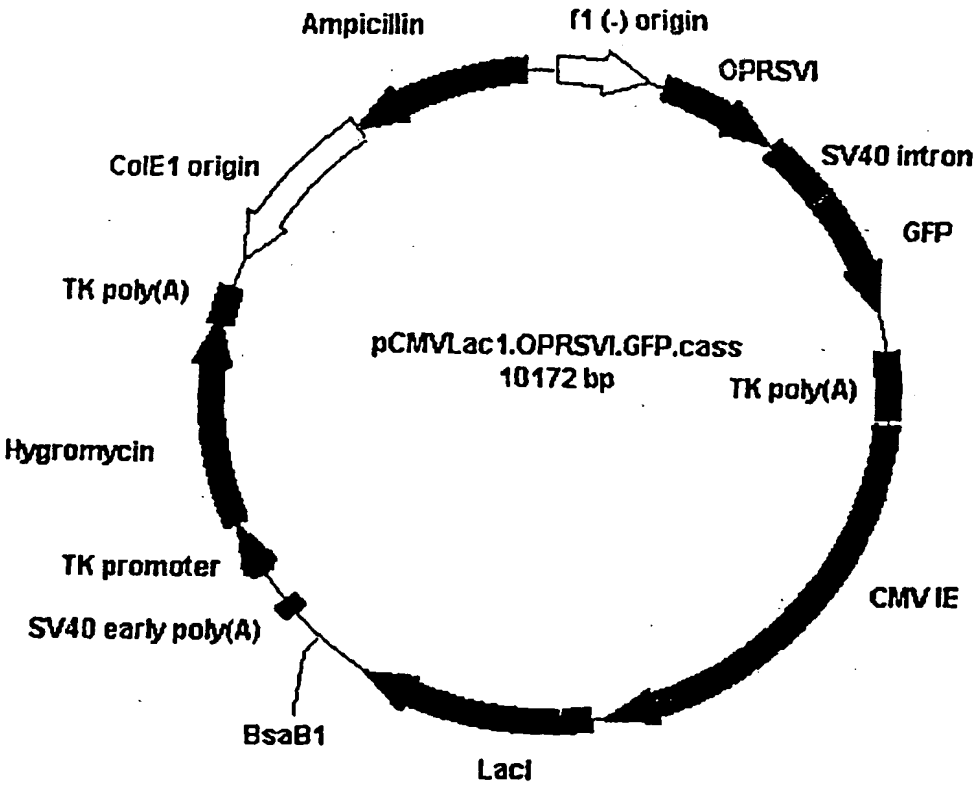

Ampicillin
f1 (-) origin
OPRSVI
SV40 intron
GFP
TK poly(A)
pCMVLac1.OPRSVI.GFP.cass
10172 bp
TK poly(A)
CMV IE
LacI
BsaB1
SV40 early poly(A)
TK promoter
Hygromycin
ColE1 origin

FIGURE 27

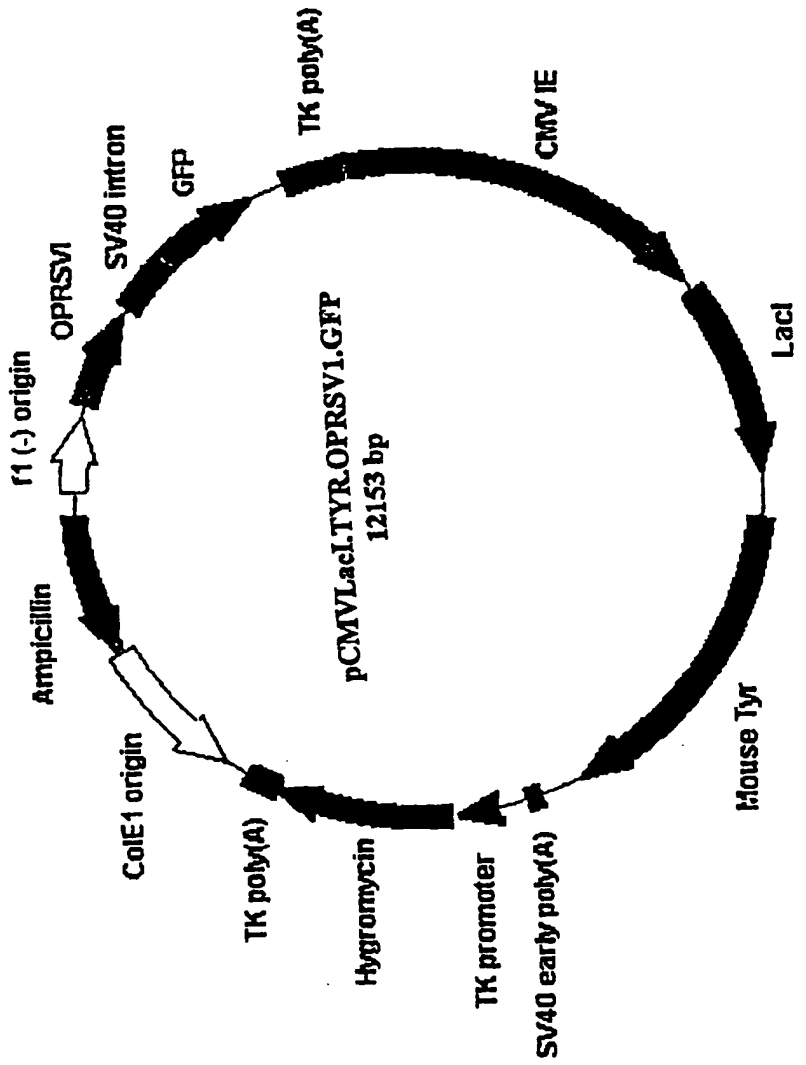
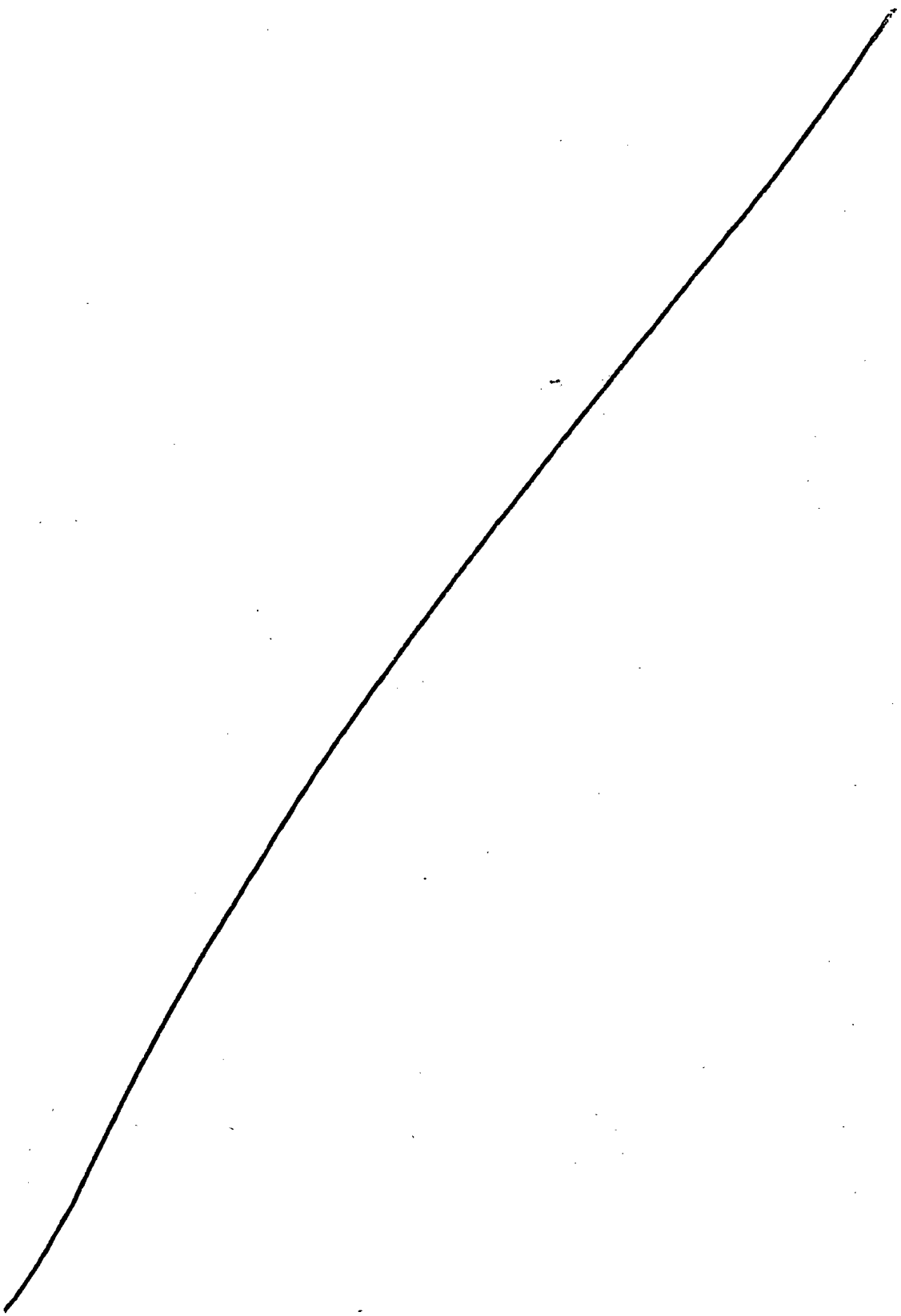

pCMVLacI.TYR.OPRSV1.GFP
12153 bp
TK poly(A)
CMV IE
LacI
Mouse Tyr
SV40 early poly(A)
TK promoter
Hygromycin
TK poly(A)
ColE1 origin
Ampicillin
f1 (-) origin
OPRSVI
SV40 intron
GFP

FIGURE 28



Printed: 22-04-2005 TIPA1 99910592

PCT/AU99/00195

REC'D 20 APR 1999
WIPO PCT

5

EPO - DG 1
20. 04. 2005
(93)

Patent Office
Canberra

I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PP 2499 for a patent by AG-GENE AUSTRALIA LTD and STATE OF QUEENSLAND THROUGH ITS DEPARTMENT OF PRIMARY INDUSTRIES filed on 20 March 1998.

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

WITNESS my hand this Fourteenth day of April 1999

KIM MARSHALL
MANAGER EXAMINATION SUPPORT AND SALES

384
20-04-2005

Printed: 22-04-2005 TIPA 99910592

Regulation 3.2

AUSTRALIAN
PROVISIONAL No. FILING
PP2493 20 MAR. 98
PATENT OFFICE

Ag-Gene Australia Ltd

AND

State of Queensland through its Department of Primary Industries

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Gene Expression I"

The invention is described in the following statement:

Printed: 20-04-2005

Printed: 22-04-2005 TIPA 99910592

GENE EXPRESSION I

FIELD OF THE INVENTION

The present invention relates generally to a method of modifying gene expression and reagents therefor. More particularly, the present invention utilises recombinant DNA technology to post-transcriptionally modify or modulate the expression of a target gene in a cell, tissue, organ or whole organism, thereby producing novel phenotypes.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

BACKGROUND TO THE INVENTION

Controlling metabolic pathways in eukaryotic organisms is desirable for the purposes of producing novel traits therein or introducing novel traits into a particular cell, tissue or organ of said organism. Whilst recombinant DNA technology has provided significant progress in an understanding of the mechanisms regulating eukaryotic gene expression, much less progress has been made in the actual manipulation of gene expression to produce novel traits. Moreover, there are only limited means by which human intervention may lead to a modulation of the level of eukaryotic gene expression.

SISIS 20-04-2005

Printed: 22-04-2005 IPAU 99910592

One approach to repressing, delaying or otherwise reducing gene expression utilise a mRNA molecule which is transcribed from the complementary strand of a nuclear gene to that which is normally transcribed and capable of being translated into a polypeptide. Although the precise mechanism involved in this approach is not established, it has been postulated that a double-stranded mRNA may form by base pairing between the complementary nucleotide sequences, to produce a complex which is translated at low efficiency and/or degraded by intracellular ribonuclease enzymes prior to being translated.

Alternatively, the expression of an endogenous gene in a cell, tissue or organ may be suppressed when one or more copies of said gene, or one or more copies of a substantially similar gene are introduced into the cell. Whilst the mechanism involved in this phenomenon has not been established and appears to be involve mechanistically heterogeneous processes. For example, this approach has been postulated to involve transcriptional repression, in which case somatically-heritable repressed states of chromatin are formed or alternatively, a post-transcriptional silencing wherein transcription initiation occurs normally but the RNA products of the co-suppressed genes are subsequently eliminated.

The efficiency of both of these approaches in targeting the expression of specific genes is very low and highly variable results are usually obtained. Inconsistent results are obtained using different regions of genes, for example 5'- untranslated regions, 3'-untranslated regions, coding regions or intron sequences to target gene expression. Accordingly, there currently exists no consensus as to the nature of genetic sequences which provide the most efficient means for repressing, delaying or otherwise reducing gene expression using existing technologies. Moreover, such a high degree of variation exists between generations such that it is not possible to predict the level of repression of a specific gene in the progeny of an organism in which gene expression was markedly modified.

Recently, Dorer and Henikoff (1994) demonstrated the silencing of tandemly repeated gene copies in the *Drosophila* genome and the transcriptional repression of dispersed *Drosophila*

20-04-2005

Printed: 22-04-2005 TIPAT 99910592

Adh genes by *Polycomb* genes (i.e. the *Pc-G* system; Pal-Bhadra *et al*, 1997). However, such silencing of tandemly repeated gene copies is of little utility in an attempt to manipulate gene expression in an animal cell by recombinant means, wherein the sequences capable of targeting the expression of a particular gene are introduced at dispersed locations in the genome, absent the combination of this approach with gene-targeting technology. Whilst theoretically possible, such combinations would be expected to work at only low-efficiency, based upon the low efficiency of gene-targeting approaches used in isolation and further, would require complicated vector systems. Additionally, the utilisation of transcriptional repression, such as the *Drosophila Pc-G* system, would appear to require some knowledge of the regulatory mechanisms capable of modulating the expression of any specific target gene and, as a consequence, would be difficult to implement in practice as a general technology for repressing, delaying or reducing gene expression in animal cells.

The poor understanding of the mechanisms involved in these phenomena has meant that there have been few improvements in technologies for modulating the level of gene expression , in particular technologies for delaying, repressing or otherwise reducing the expression of specific genes using recombinant DNA technology. Furthermore, as a consequence of the unpredictability of these approaches, there is currently no commercially-viable means for modulating the level of expression of a specific gene in a eukaryotic or prokaryotic organism.

Thus, there exists a need for improved methods of modulating gene expression, in particular repressing, delaying or otherwise reducing gene expression in animal cells for the purpose of introducing novel phenotypic traits thereto. In particular, these methods should provide general means for phenotypic modification, without the necessity for performing concomitant gene-targeting approaches.

SUMMARY OF THE INVENTION

The invention is based in part on the surprising discovery by the inventors that cells which exhibit one or more desired traits can be produced and selected from transformed cells comprising a nucleic acid molecule operably linked to a promoter, wherein the transcription

20-04-2005

Printed: 22-04-2005

TIPAT

99910592

product of the nucleic acid molecule comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of a transcript of an endogenous or non-endogenous target gene, the expression of which is intended to be modulated. The transformed cells are regenerated into whole tissues, organs or organisms capable of exhibiting novel traits, in particular virus resistance and modified expression of endogenous genes.

Accordingly, the present invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the step of introducing to said cell, tissue or organ one or more dispersed nucleic acid molecules or foreign nucleic acid molecules comprising a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or complementary thereto for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

In a particularly preferred embodiment, the dispersed nucleic acid molecules or foreign nucleic acid molecules comprises a nucleotide sequence which encodes an mRNA molecule which is substantially identical to the mRNA product of the target gene.

In a more particularly preferred embodiment, the dispersed nucleic acid molecule or foreign nucleic acid molecule is in an expressible form such that it is at least capable of being transcribed to produce mRNA.

The target gene may be a gene which is endogenous to the animal cell or alternatively, a foreign gene such as a viral or foreign genetic sequence, amongst others. Preferably, the target gene is a viral genetic sequence.

The invention is particularly useful in the modulation of eukaryotic gene expression, in particular the modulation of human or animal gene expression and even more particularly in the modulation of expression of genes derived from vertebrate and invertebrate animals, such

20-04-2005

as insects, aquatic animals (eg. fish, shellfish, molluscs, crustaceans such as crabs, lobsters and prawns, avian animals and mammals, amongst others).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a copy of a diagrammatic representation of the plasmid pEGFP.BEV.1.

Figure 2 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.2.

Figure 3 is a copy of a diagrammatic representation of the plasmid pCMV.VEB.

Figure 4 is a copy of a diagrammatic representation of the plasmid pCMV.BEVnt.

Figure 5 is a copy of a diagrammatic representation of the plasmid pCMV.BEVx2.

Figure 6 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.VEB.

Figure 7 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.GFP.VEB.

Figure 8 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.SV40L-0.

Figure 9 is a copy of a diagrammatic representation of the plasmid pCMV.0.SV40L.BEV.

Figure 10 is a copy of a diagrammatic representation of the plasmid pCMV.0.SV40L.VEB.

Figure 11 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.SV40L.BEV.

Figure 12 is a copy of a diagrammatic representation of the plasmid

pCMV.BEV.SV40L.VEB.

Figure 13 is a copy of a diagrammatic representation of the plasmid pCMV.SV40LR.cass.

Figure 14 is a copy of a diagrammatic representation of the plasmid pCMV.BEV.SV40LR.

Figure 15 is a copy of a diagrammatic representation of the plasmid pCMV.TYR.

Figure 16 is a copy of a diagrammatic representation of the plasmid pCMV.TYRLIB.

Figure 17 is a copy of a diagrammatic representation of the plasmid pCMVLacI.OPRSV1.GFP.TYR.

Figure 18 is a copy of a diagrammatic representation of the plasmid pCMVLacI.OPRSV1.GFP.cass.

Figure 19 is a copy of a diagrammatic representation of the plasmid pCMV.Lac.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the step of introducing to said cell, tissue or organ one or more dispersed nucleic acid molecules or foreign nucleic acid molecules comprising a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or complementary thereto for a time and

under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

As used herein, the term "modulating" shall be taken to mean that expression of the target gene is either increased or reduced in amplitude or the timing of gene expression or the developmental or tissue-specific or cell-specific pattern of target gene expression is altered, compared to the expression of said gene in the absence of the inventive method described herein.

In a preferred embodiment, whilst not limiting the scope of the invention described herein, the present invention is directed to a modulation of gene expression which comprises the repression, delay or reduction in amplitude of target gene expression in a specified cell, tissue or organ of a eukaryotic organism, in particular a human or other animal and even more particularly a vertebrate and invertebrate animal, such as an insect, aquatic animal (eg. fish, shellfish, mollusc, crustacean such as a crab, lobster or prawn, an avian animal or a mammal, amongst others).

More preferably, target gene expression is completely inactivated by the dispersed nucleic acid molecules or foreign nucleic acid molecules which has been introduced to the cell, tissue or organ.

Whilst not being bound by any theory or mode of action, the reduced or eliminated expression of the target gene which results from the performance of the invention may be attributed to reduced or delayed translation of the mRNA transcription product of the target gene or alternatively, the prevention of translation of said mRNA, as a consequence of sequence-specific degradation of the mRNA transcript of the target gene by an endogenous host cell system.

It is particularly preferred that, for optimum results, sequence-specific degradation of the

mRNA transcript of the target gene occurs either prior to the time or stage when the mRNA transcript of the target gene would normally be translated or alternatively, at the same time as the mRNA transcript of the target gene would normally be translated. Accordingly, the selection of an appropriate promoter sequence to regulate expression of the introduced dispersed nucleic acid molecule or foreign nucleic acid molecule is an important consideration to optimum performance of the invention. For this reason, strong constitutive promoters or inducible promoter systems are especially preferred for use in regulating expression of the introduced dispersed nucleic acid molecules or foreign nucleic acid molecules.

The present invention clearly encompasses reduced expression wherein reduced expression of the target gene is effected by lowered transcription, subject to the proviso that a reduction in transcription is not the sole mechanism by which this occurs and said reduction in transcription is at least accompanied by reduced translation of the steady-state mRNA pool.

The target gene may be a genetic sequence which is endogenous to the animal cell or alternatively, a non-endogenous genetic sequence, such as a genetic sequence which is derived from a virus or other foreign pathogenic organism and is capable of entering a cell and using the cell's machinery following infection.

Wherein the target gene is a non-endogenous genetic sequence to the animal cell, it is desirable that the target gene encodes a function which is essential for replication or reproduction of the viral or other pathogen. In such embodiments, the present invention is particularly useful in the prophylactic and therapeutic treatment of viral infection of an animal cell or for conferring or stimulating resistance against said pathogen.

More preferably, the target gene comprises one or more nucleotide sequences of a viral pathogen of an animal cell, tissue or organ, such as but not limited to a retrovirus, for example a lentivirus such as the immunodeficiency viruses, a single-stranded (+) RNA virus such as bovine enterovirus (BEV) or Sinbis alphavirus. Alternatively, the target gene comprises one or more nucleotide sequences of a viral pathogen of an animal cell, tissue or

organ, such as but not limited to a double-stranded DNA virus such as bovine herpes virus or herpes simplex virus I (HSV I), amongst others.

With particular regard to viral pathogens, those skilled in the art are aware that virus-encoded functions may be complemented *in trans* by polypeptides encoded by the host cell. For example, the replication of the bovine herpes virus genome in the host cell may be facilitated by host cell DNA polymerases which are capable of complementing an inactivated viral DNA polymerase gene.

Accordingly, wherein the target gene is a non-endogenous genetic sequence to the animal cell, a further alternative embodiment of the invention provides for the target gene to encode a viral or foreign polypeptide which is not capable of being complemented by a host cell function, such as a virus-specific genetic sequence. Exemplary target genes according to this embodiment of the invention include, but are not limited to genes which encode virus coat proteins, uncoating proteins and RNA-dependent DNA polymerases and RNA-dependent RNA polymerases, amongst others.

In a particularly preferred embodiment of the present invention, the target gene is the BEV RNA-dependent RNA polymerase gene or a homologue, analogue or derivative thereof.

The animal cell in which expression of the target gene is modified may be any cell which is derived from a multicellular animal, including cell and tissue cultures thereof. Preferably, the animal cell is derived from an insect, reptile, amphibian, bird, human or other mammal. Exemplary animal cells include embryonic stem cells, cultured skin fibroblasts, neuronal cells, somatic cells, haematopoietic stem cells, T-cells and immortalised cell lines such as COS, VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI-38, baby hamster kidney (BHK) or MDBK cell lines, amongst others. Such cells and cell lines are readily available to those skilled in the art. Accordingly, the tissue or organ in which expression of the target gene is modified may be any tissue or organ comprising such animal cells.

Printed: 22-04-2005 TIPAT 99910592

As used herein, the term "dispersed nucleic acid molecule" shall be taken to refer to a nucleic acid molecule which comprises a nucleotide sequence which is substantially identical or complementary to the nucleotide sequence of a gene which originates from the cell, tissue or organ into which said nucleic acid molecule is introduced, wherein said nucleic acid molecule is non-endogenous in the sense that it is introduced to the cell, tissue or organ of an animal via recombinant means and will generally be present as extrachromosomal nucleic acid or alternatively, as integrated chromosomal nucleic acid which is genetically-unlinked to said gene. More particularly, the "dispersed nucleic acid molecule" will comprise chromosomal or extrachromosomal nucleic acid which is unlinked to the target gene against which it is directed in a physical map, by virtue of their not being tandemly-linked or alternatively, occupying a different chromosomal position on the same chromosome or being localised on a different chromosome or present in the cell as an episome, plasmid, cosmid or virus particle.

By "foreign nucleic acid molecule" is meant an isolated nucleic acid molecule which has a nucleotide sequence which originates from the genetic sequence of an organism which is different from the organism to which the foreign nucleic acid molecule is introduced. This definition encompasses a nucleic acid molecule which originates from a different individual of the same lowest taxonomic grouping (i.e. the same population) as the taxonomic grouping to which said nucleic acid molecule is introduced, as well as a nucleic acid molecule which originates from a different individual of a different taxonomic grouping as the taxonomic grouping to which said nucleic acid molecule is introduced.

Accordingly, a target gene which comprises a foreign nucleic acid molecule may be a nucleic acid molecule which has been introduced from one organism to another organism using transformation or introgression technologies. Exemplary foreign nucleic acid molecules according to this embodiment of the invention include the green fluorescent protein-encoding gene derived from the jellyfish *Aequoria victoria* (Prasher *et al.*, 1992; International Patent Publication No. WO 95/07463), tyrosinase genes and in particular the murine tyrosinase gene (Kwon *et al.*, 1988), the *Escherichia coli lacI* gene which is capable of encoding a polypeptide

SUBSTITUTE SHEET (RULE 26) 20-04-2005

repressor of the *lacZ* gene or a homologue, analogue or derivative of said genes or a complementary nucleotide sequence thereto.

The present invention is further useful for simultaneously targeting the expression of several target genes which are co-expressed in a particular cell, for example by using a dispersed nucleic acid molecule or foreign nucleic acid molecule which comprises nucleotide sequences which are substantially identical to each of said co-expressed target genes.

By "substantially identical" is meant that the introduced dispersed or foreign nucleic acid molecule of the invention and the target gene sequence are sufficiently identical at the nucleotide sequence level to permit base-pairing there between under standard intracellular conditions.

Preferably, the dispersed or foreign nucleic acid molecule of the invention and the target gene sequence are at least about 80-85% identical at the nucleotide sequence level, more preferably at least about 85-90% identical, even more preferably at least about 90-95% identical and still even more preferably at least about 95-99% or 100% identical at the nucleotide sequence level to the target gene.

Preferably, the dispersed or foreign nucleic acid molecule which is introduced to the cell, tissue or organ comprises RNA or DNA.

More preferably, the dispersed or foreign nucleic acid molecule further comprises a nucleotide sequence or is complementary to a nucleotide sequence which is capable of encoding an amino acid sequence encoded by the target gene. Even more preferably, the nucleic acid molecule includes one or more ATG or AUG translational start codons.

In a particularly preferred embodiment of the invention, the dispersed or foreign nucleic acid molecule comprises a nucleotide sequence which is derived from the open reading frame of a viral polymerase gene, such as a retrovirus, lentivirus, Sinbis alphavirus or bovine

enterovirus (BEV) RNA-dependent RNA polymerase, HSV I DNA polymerase or bovine herpes virus DNA polymerase, amongst others or alternatively, a viral coat protein gene, such as the Sinbis alphavirus, bovine enterovirus, HSV I or bovine herpes virus coat protein genes.

In an alternative embodiment, the foreign or dispersed nucleic acid molecule comprises a nucleotide sequence which is derived from the murine tyrosinase gene.

In a further alternative embodiment, the foreign or dispersed nucleic acid molecule comprises a nucleotide sequence which is derived from the *E. coli lac*I gene.

In a further alternative embodiment, the foreign or dispersed nucleic acid molecule comprises a nucleotide sequence which is derived from the *A. victoria* GFP gene.

In still a further alternative embodiment of the invention, the foreign or dispersed nucleic acid molecule comprises one or more nucleotide sequences which are derived from the bovine enterovirus (BEV) RNA-dependent RNA polymerase gene and a nucleotide sequence which is derived from the *A. victoria* GFP gene in a single genetic construct and capable of being transcribed into a single mRNA molecule.

In yet still a further alternative embodiment of the invention, the foreign or dispersed nucleic acid molecule comprises one or more nucleotide sequences which are derived from the murine tyrosinase gene and the *E. coli lac*I gene in a single genetic construct and capable of being transcribed into a single mRNA molecule. Optionally, the foreign or dispersed nucleic acid molecule further comprises a nucleotide sequence which is derived from the *A. victoria* GFP gene.

The present invention is not in any way limited by the precise nucleotide sequence of the foreign or dispersed nucleic acid molecule or by the number or configuration of target gene sequences therein which is/are used to modulate expression of the target gene and all such

sequences are referred to herein merely for the purposes of exemplification to demonstrate the efficacy of the inventive method.

Standard methods may be used to introduce the dispersed nucleic acid molecule or foreign nucleic acid molecule into the cell, tissue or organ for the purposes of modulating the expression of the target gene. For example, the nucleic acid molecule may be introduced as naked DNA or RNA, optionally encapsulated in a liposome, in a virus particle as attenuated virus or associated with a virus coat or a transport protein or inert carrier such as gold or as a recombinant viral vector or bacterial vector or as a genetic construct, amongst others.

Administration means include injection and oral ingestion (e.g. in medicated food material), amongst others.

The subject nucleic acid molecules may also be delivered by a live delivery system such as using a bacterial expression system optimised for their expression in bacteria which can be incorporated into gut flora. Alternatively, a viral expression system can be employed. In this regard, one form of viral expression is the administration of a live vector generally by spray, feed or water where an infecting effective amount of the live vector (e.g. virus or bacterium) is provided to the animal. Another form of viral expression system is a non-replicating virus vector which is capable of infecting a cell but not replicating therein. The non-replicating viral vector provides a means of introducing to the human or animal subject genetic material for transient expression therein. The mode of administering such a vector is the same as a live viral vector.

The carriers, excipients and/or diluents utilised in delivering the subject nucleic acid molecules to a host cell should be acceptable for human or veterinary applications. Such carriers, excipients and/or diluents are well-known to those skilled in the art. Carriers and/or diluents suitable for veterinary use include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. Except insofar as any conventional media or agent is incompatible with the active

ingredient, use thereof in the composition is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

In an alternative embodiment, the invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the steps of:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or which is complementary thereto; and

(ii) introducing said dispersed nucleic acid molecules or foreign nucleic acid molecules to said cell, tissue or organ for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

To select appropriate nucleotide sequences for targeting expression of the target gene, several approaches may be employed. In one embodiment, specific regions of characterised genes may be cloned in operable connection with a suitable promoter and assayed for efficacy in reducing target gene expression in animal cells. Alternatively, shotgun libraries of genetic sequences may be produced and assayed for their efficacy in reducing target gene expression. The advantage associated with the latter approach is that it is not necessary to have any prior knowledge of the significance of any particular target gene in specifying an undesirable phenotype in the animal cell. For example, shotgun libraries comprising virus sub-genomic fragments may be employed and tested directly for their ability to confer virus immunity on the animal host cell, without prior knowledge of the role which any virus genes play in pathogenesis of the host cell.

As used herein, the term "shotgun library" is a set of diverse nucleotide sequences wherein each member of said set is preferably contained within a suitable plasmid, cosmid,

Printed: 22-04-2005 TIPA1 99910592

bacteriophage or virus vector molecule which is suitable for maintenance and/or replication in a cellular host. The term "shotgun library" includes a representative library, in which the extent of diversity between the nucleotide sequences is numerous such that all sequences in the genome of the organism from which said nucleotide sequences is derived are present in the "set" or alternatively, a limited library in which there is a lesser degree of diversity between said sequences. The term "shotgun library" further encompasses random nucleotide sequences, wherein the nucleotide sequence comprises viral or cellular genome fragments, amongst others obtained for example by shearing or partial digestion of genomic DNA using restriction endonucleases, amongst other approaches. A "shotgun library" further includes cells, virus particles and bacteriophage particles comprising the individual nucleotide sequences of the diverse set.

Preferred shotgun libraries according to this embodiment of the invention are "representative libraries", comprising a set of nucleotide sequences derived from a viral pathogen of an animal or alternatively, derived from an animal cell.

In a particularly preferred embodiment of the invention, the shotgun library comprises cells, virus particles or bacteriophage particles comprising a diverse set of nucleotide sequences which encode a diverse set of amino acid sequences, wherein the member of said diverse set of nucleotide sequences are placed operably under the control of a promoter sequence which is capable of directing the expression of said nucleotide sequence in an animal cell.

Accordingly, the nucleotide sequence may comprise at least about 1 to 200 nucleotides in length. The use of larger fragments, particularly employing randomly sheared nucleic acid derived from viral or animal genomes, is not excluded.

The introduced nucleic acid molecule is preferably in an expressible form.

By "expressible form" is meant that the subject nucleic acid molecule is presented in an arrangement such that it may be expressed in an animal cell, tissue, organ or whole organism,

400 20-04-2005

at least at the transcriptional level (i.e. it is expressed in the animal cell to yield at least an mRNA product which is optionally translatable or translated to produce a recombinant peptide, oligopeptide or polypeptide molecule).

By way of exemplification, in order to obtain expression of the dispersed nucleic acid molecule or foreign nucleic acid molecule in the cell, tissue or organ of interest a synthetic gene or a genetic construct comprising said synthetic gene is produced, wherein said synthetic gene comprises a nucleotide sequence as described *supra* in operable connection with a promoter sequence which is capable of regulating expression therein. Thus, the subject nucleic acid molecule will be operably connected to one or more regulatory elements sufficient for eukaryotic transcription to occur.

Accordingly, a further alternative embodiment of the invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the steps of:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or which is complementary thereto;

(ii) producing a synthetic gene comprising said dispersed nucleic acid molecules or foreign nucleic acid molecules or a genetic construct comprising same;

(iii) introducing said synthetic gene or genetic construct to said cell, tissue or organ; and

(iv) expressing said synthetic gene or genetic construct in said cell, tissue or organ for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

Hereinafter, the term "structural gene region" shall be taken to refer to that part of a synthetic gene which comprises a dispersed nucleic acid molecule or foreign nucleic acid molecule as

described herein which is expressed in a cell, tissue or organ under the control of a promoter sequence to which it is operably connected. A structural gene region may comprise one or more dispersed nucleic acid molecules and/or foreign nucleic acid molecules operably under the control of a single promoter sequence or multiple promoter sequences. Accordingly, the structural gene region of a synthetic gene may comprise a nucleotide sequence which is capable of encoding an amino acid sequence or is complementary thereto. In this regard, a structural gene region which is used in the performance of the instant invention may also comprise a nucleotide sequence which encodes an animo acid sequence yet lacks a functional translation initiation codon and/or a functional translation stop codon and, as a consequence, does not comprise a complete open reading frame. In the present context, the term "structural gene region" also extends to a non-coding nucleotide sequences, such as 5'- upstream or 3'- downstream sequences of a gene which would not normally be translated in a eukaryotic cell which expresses said gene.

Wherein the structural gene region comprises more than one dispersed nucleic acid molecule or foreign nucleic acid molecule it shall be referred to herein as a "multiple structural gene region" or similar term. The present invention clearly extends to the use of multiple structural gene regions which preferably comprise a direct repeat sequence, inverted repeat sequence or interrupted palindrome sequence of a particular structural gene or a fragment thereof.

Reference herein to a "gene" or "genes" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences); and/or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5'- and 3'- untranslated sequences of the gene; and/or

(iii) a structural region corresponding to the coding regions (i.e. exons) optionally further comprising untranslated sequences and/or a heterologous promoter sequence which consists of transcriptional and/or translational regulatory regions capable of conferring expression

Printed: 22-04-2005 TIPAT 99910592

characteristics on said structural region.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product.

Accordingly, in the context of the present invention, a structural gene region may also comprise a fusion between two or more open reading frames of the same or different genes. In such embodiments, the invention may be used to modulate the expression of one gene, by targeting different non-contiguous regions thereof or alternatively, to simultaneously modulate the expression of several different genes, including different genes of a multigene family. In the case of a fusion nucleic acid molecule which is non-endogenous to the animal cell and in particular comprises two or more nucleotide sequences derived from a viral pathogen, the fusion may provide the added advantage of conferring simultaneous immunity or protection against several pathogens, by targeting the expression of genes in said several pathogens. Alternatively or in addition, the fusion may provide more effective immunity against any pathogen by targeting the expression of more than one gene of that pathogen.

Particularly preferred structural gene regions according to this aspect of the invention are those which include at least one translatable open reading frame, more preferably further including a translational start codon located at the 5'-end of said open reading frame, albeit not necessarily at the 5'-terminus of said structural gene region. In this regard, notwithstanding that the structural gene region may comprise at least one translatable open reading frame and/or AUG or ATG translational start codon, the inclusion of such sequences in no way suggests that the present invention requires translation of the introduced nucleic acid molecule to occur in order to modulate the expression of the target gene. Whilst not being bound by any theory or mode of action, the inclusion of at least one translatable open reading frame and/or translational start codon in the subject nucleic acid molecule may serve to increase stability of the mRNA transcription product thereof, thereby improving the efficiency of the invention.

20-04-2005

Wherein the synthetic gene or genetic construct is expressed, it is particularly preferred that the structural gene region or multiple structural gene region thereof comprising the dispersed or foreign nucleic acid molecule is expressed prior to expression of the target gene or alternatively, simultaneous with transcription of the mRNA product of the target gene.

Depending on the duration and relative levels of expression of the synthetic gene and target gene, an animal cell carrying both sequences will exhibit a variety of different phonotypic traits. In particular, selecting cells, tissues or organs which express varying phenotypes can be readily achieved in accordance with the present invention.

In consideration of the preferred requirement for high-level expression which coincides with expression of the target gene or precedes expression of the target gene, it is highly desirable that the promoter sequence is a constitutive strong promoter such as the CMV-IE promoter or the SV40 early promoter sequence or SV40 late promoter sequence, amongst others.

For expression in eukaryotic cells, the synthetic gene or genetic construct generally comprises, in addition to the nucleic acid molecule of the invention, a promoter and optionally other regulatory sequences designed to facilitate expression of the dispersed nucleic acid molecule or foreign nucleic acid molecule.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5', of a structural gene region, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene.

Printed: 22-04-2005

TIPAT

99910592

P:\OPER\MRO\AO-GENE1.PRV - 15/1/98

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression of the sense molecule and/or to alter the spatial expression and/or temporal expression of said sense molecule. For example, regulatory elements which confer copper inducibility may be placed adjacent to a heterologous promoter sequence driving expression of a sense molecule, thereby conferring copper inducibility on the expression of said molecule.

Placing a nucleic acid molecule under the regulatory control of a promoter sequence means positioning the said molecule such that expression is controlled by the promoter sequence. Promoters are generally positioned 5' (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in producing synthetic genes or genetic constructs include viral, fungal, bacterial, animal and plant derived promoters capable of functioning in animal cell, in particular a human, insect, fish, crustacean, mollusc, avian, reptile or mammalian cell. The promoter may regulate the expression of the said molecule constitutively, or differentially with respect to the tissue in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external

405

20-04-2005

stimuli such as physiological stresses, or plant pathogens, or metal ions, amongst others.

Preferably, the promoter is capable of regulating expression of a structural gene region or multiple structural gene region in a cell derived from an mammal or a human.

In a particularly preferred embodiment, the promoter is the CMV-IE or the SV40 early promoter sequence or SV40 late promoter sequence or the RSV1 LTR promoter or a promoter sequence derived therefrom.

In a preferred embodiment of the invention, a structural gene region or multiple structural gene region is placed in the "sense" orientation relative to the promoter sequence, such that when it is transcribed an mRNA product is synthesized which, if translated, is capable of encoding a polypeptide product of the target gene or a fragment thereof.

However, the present invention is not to be limited to the use of such an arrangement and the invention clearly extends to the use of synthetic genes and genetic constructs wherein the a structural gene region or multiple structural gene region is placed in the "antisense" orientation relative to the promoter sequence, such that at least a part of the mRNA transcription product thereof is complementary to the mRNA encoded by the target gene or a fragment thereof.

In an alternative embodiment of the invention, the structural gene region or multiple structural gene region is operably connected to both a first promoter sequence and a second promoter sequence, wherein said promoters are located at the distal and proximal ends thereof such that said a structural gene region or multiple structural gene region is placed in the "sense" orientation relative to the first promoter sequence and in the "antisense" orientation relative to the second promoter sequence. According to this embodiment, it is also preferred that the first and second promoters be different, to prevent competition there between for cellular transcription factors which bind thereto. The advantage of this arrangement is that the effects of transcription from the first and second promoters in reducing target gene expression in the

cell may be compared to determine the optimum orientation for each nucleotide sequence tested.

In a further alternative embodiment, a genetic construct is used which comprises two or more structural gene regions or multiple structural gene regions wherein each of said structural gene regions is placed operably under the control of its own promoter sequence. As with other embodiments described herein, the orientation of each structural gene region may be varied to maximise its modulatory effect on target gene expression.

The synthetic gene or genetic construct comprising same preferably contains additional regulatory elements for efficient transcription, for example a transcription termination sequence.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in plant cells are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants or synthesized *de novo*.

As with promoter sequences, the terminator may be any terminator sequence which is operable in the cells, tissues or organs in which it is intended to be used.

Particularly preferred terminators for use in the genetic constructs include mammalian cell-expressible terminator sequences such as the SPA terminator sequence, CMV-IE gene terminator and SV40 large T antigen gene terminator amongst others.

Those skilled in the art will be aware of how to produce the synthetic genes and genetic constructs described herein and of the requirements for obtaining the expression thereof, when so desired, in a specific cell or cell-type under the conditions desired. In particular,

it will be known to those skilled in the art that the genetic manipulations required to perform the present invention may require the propagation of a genetic construct described herein or a derivative thereof in a prokaryotic cell such as an *E. coli* cell or an animal cell.

As will be known to those skilled in the art, genetic constructs may further comprise genetic sequences corresponding to a bacterial origin of replication and/or a selectable marker gene such as an antibiotic-resistance gene, suitable for the maintenance and replication of said genetic construct in a prokaryotic or eukaryotic organism. Such sequences are well-known in the art. Usually, an origin of replication or a selectable marker gene suitable for use in bacteria is physically-separated from those genetic sequences contained in the genetic construct which are intended to be expressed or transferred to a eukaryotic cell, or integrated into the genome of a eukaryotic cell.

Means for transfecting or transforming animal cells with the synthetic genes described herein or a genetic construct comprising same are well-known to those skilled in the art.

The present invention extends to all genetic constructs essentially as defined herein, which include further genetic sequences intended for the maintenance and/or replication of said genetic construct in prokaryotes or eukaryotes and/or the integration of said genetic construct or a part thereof into the genome of a eukaryotic cell or organism.

As with dispersed or foreign nucleic acid molecules, standard methods described *supra* may be used to introduce synthetic genes and genetic constructs into the cell, tissue or organ for the purposes of modulating the expression of the target gene. Particularly, preferred methods suited to the introduction of synthetic genes and genetic constructs comprising same to eukaryotic cells include liposome-mediated transfection or transformation, transformation of cells with attenuated virus particles or bacterial cells and standard procedures for the transformation of animal cells, tissues, organs or organisms.

Genetic constructs are particularly suitable for the transformation of a eukaryotic cell to

introduce novel genetic traits thereto, in addition to the provision of resistance characteristics to viral pathogens. Such additional novel traits may be introduced in a separate genetic construct or, alternatively on the same genetic construct which comprises the synthetic genes described herein. Those skilled in the art will recognise the significant advantages, in particular in terms of reduced genetic manipulations and tissue culture requirements and increased cost-effectiveness, of including genetic sequences which encode such additional traits and the synthetic genes described herein in a single genetic construct.

In a further embodiment of the present invention, the synthetic genes and genetic constructs described herein are adapted for integration into the genome of a cell in which it is expressed. Those skilled in the art will be aware that, in order to achieve integration of a genetic sequence or genetic construct into the genome of a host cell, certain additional genetic sequences may be required.

The present invention clearly extends to isolated cells carrying the genetic constructs of the invention and to regenerated tissues, organs and whole organisms derived therefrom.

A variety of traits are selectable with appropriate procedures and sufficient numbers of transformed cells. Such traits include, but are not limited to, visible traits, disease-resistance traits, and pathogen-resistance traits. The modulatory effect is applicable to a variety of genes expressed in animals including, for example, endogenous genes responsible for cellular metabolism or cellular transformation, including oncogenes, transcription factors and other genes which encode polypeptides involved in cellular metabolism.

For example, an alteration in the pigment production in mice is engineered by targeting the expression of the tyrosinase gene therein. This provides a novel phenotype of albinism in black mice. Additionally, by targeting genes required for virus replication in an animal cell, the genetic construct of the instant invention which comprises a virus replicase, polymerase, coat protein or uncoating gene may be introduced into a cell where it is expressed, to confer immunity against the virus upon the cell.

Among the easiest genes to target for the performance of the invention are the viral pathogen genes, which are utilised to confer resistance on the animal cell, because of the smaller genome size of such organisms compared to animals. In particular, the smaller genome size means that small sub-genomic libraries are required to be produced to qualify as "representative libraries".

The introduced sequence generally will be substantially homologous to the endogenous sequence intended to be modulated, such that the controlling elements recognize that the introduced sequence is present, the interaction results in the modulatory effect. This minimal homology will typically be greater than about 85%, but a higher homology might exert a more effective modulation of expression of the endogenous sequences. Substantially greater homology, or more than about 90% is preferred, though about 95% to absolute identity would be most preferred. Consequently, the effect should apply to any other proteins within a similar family of genes exhibiting homology or substantial homology.

The introduced sequence, needing less than absolute homology, also need not be full length, relative to either the primary transcription product or fully processed mRNA. A higher homology in a shorter than full length sequence compensates for a longer less homologous sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments will be equally effective. Normally, a sequence of greater than 20-100 nucleotides should be used, though a sequence of greater than about 200-300 nucleotides would be preferred, and a sequence of greater than 500-1000 nucleotides would be especially preferred depending on the size of the endogenous gene.

In order to observe many novel traits, in particular those which are tissue-specific or organ-specific or developmentally-regulated, regeneration of a transformed cell carrying the synthetic genes and genetic constructs described herein will be required. those skilled in the art will be aware that this means growing a whole organism from a transformed animal cell, a group of such cells, a tissue or organ. Standard methods for the regeneration of certain animals from isolated cells are known to those skilled in the art.

The present invention is further described with reference to the following non-limiting Examples.

EXAMPLE 1

Genetic constructs

A) Genetic constructs comprising a BEV polymerase structural gene operably connected to the CMV-IE promoter sequence.

Isolation of BEV RNA polymerase gene fragments

The BEV RNA-dependent RNA polymerase coding region was amplified as a 1,385 bp DNA fragment from a full-length cDNA clone encoding same, using the primers designated BEV-1 (SEQ ID NO:1) and BEV-2 (SEQ ID NO:2), under standard amplification conditions. The amplified DNA contained a 5'-*Bgl* II restriction enzyme site, derived from the BEV-1 primer sequence and a 3'*Bam*HI restriction enzyme site, derived from the BEV-2 primer sequence. Additionally, as the BEV-1 primer sequence contains a translation start signal 5'-ATG-3' engineered at positions 15-17 of SEQ ID NO:1, the amplified BEV polymerase structural gene comprises the start site in-frame with BEV polymerase-encoding nucleotide sequences, Thus, the amplified BEV polymerase structural gene comprises the ATG start codon immediately upstream (ie. juxtaposed) to the BEV polymerase-encoding sequence. There is no translation stop codon in the amplified DNA.

Additionally, the complete BEV polymerase coding region was amplified from a full-length cDNA clone encoding same, using primers BEV-1 (SEQ ID NO:1) and BEV-3 (SEQ ID NO:3). Primer BEV-3 comprises a *Bam*HI restriction enzyme site at positions 5 to 10 inclusive of SEQ ID NO:3 and the complement of a translation stop signal at positions 11 to 13 of SEQ ID NO:3. As a consequence, an open reading frame comprising a translation start signal and translation stop signal, contained between the *Bgl* II and *Bam*HI restriction sites.

Additionally, a non-translatable BEV polymerase structural gene was amplified from a full-

Printed: 22-04-2005 TIPA1 99910592

length BEV polymerase cDNA clone using the amplification primers BEV-3 (SEQ ID NO:3) and BEV-4 (SEQ ID NO:4). Primer BEV-4 comprises a *Bgl*II cloning site at positions 5-10 of SEQ ID NO:4 and sequences downstream of this *Bgl*II site are homologous to nucleotide sequences of the BEV polymerase gene. There is no functional ATG start codon in the amplified DNA product of primers BEV-3 and BEV-4.

1. Plasmid pEGFP.BEV.1

Plasmid pEGFP.BEV.1 (Figure 1) is capable of expressing the BEV polymerase structural gene as a GFP fusion polypeptide under the control of the CMV-IE promoter sequence.

2. Plasmid pCMV.BEV.2

Plasmid pCMV.BEV.2 (Figure 2) is capable of expressing the entire BEV polymerase open reading frame under the control of CMV-IE promoter sequence.

3. Plasmid pCMV.VEB

Plasmid pCMV.VEB (Figure 3) expresses an antisense BEV polymerase mRNA under the control of the CMV-IE promoter sequence.

4. Plasmid pCMV.BEVnt

Plasmid pCMV.BEVnt (Figure 4) expresses a non-translatable BEV polymerase structural gene in the sense orientation under the control of the CMV-IE promoter sequence.

5. Plasmid pCMV.BEVx2

Plasmid pCMV.BEVx2 (Figure 5) comprises a direct repeat of a complete BEV polymerase open reading frame under the control of the CMV-IE promoter sequence. In eukaryotic cells at least, the open reading frame located nearer the CMV-IE promoter is translatable.

6. Plasmid pCMV.BEV.VEB

Plasmid pCMV.BEV.VEB (Figure 6) comprises an inverted repeat or palindrome of a complete BEV polymerase open reading frame under the control of the CMV-IE promoter

412 20-04-2005

Filed: 22-04-2005 TIPAT 99910592

P:\OPERUM\ROVAG-GENE3 PRV - 15/3/98

sequence. In eukaryotic cells at least, the open reading frame located nearer the CMV-IE promoter is translatable.

7. Plasmid pCMV.BEV.GFP.VEB

Plasmid pCMV.BEV.GFP.VEB (Figure 7) is similar to plasmid pCMV.BEV.VEB except that the BEV structural gene inverted repeat or palindrome is interrupted by the insertion of a GFP open reading frame (stuffer fragment) therein. The BEV polymerase structural gene nearer the CMV-IE promoter sequence in plasmid pCMV.BEV.GFP.VEB is capable of being translated, at least in eukaryotic cells.

B) Genetic constructs comprising BEV polymerase structural genes operably connected to multiple promoter sequences

1. Plasmid pCMV.BEV.SV40L-O

Plasmid pCMV.BEV.SV40L-O (Figure 8) comprises a translatable BEV polymerase structural gene inserted in the sense orientation between the CMV-IE promoter and the SV40 late promoter sequences.

2. Plasmid pCMV.O.SV40L.BEV

Plasmid pCMV.O.SV40L.BEV (Figure 9) comprises a translatable BEV polymerase structural gene cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences.

3. Plasmid pCMV.O.SV40L.VEB

Plasmid pCMV.O.SV40L.VEB (Figure 10) comprises an antisense BEV polymerase structural gene cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences.

4. Plasmid pCMV.BEV.SV40L.BEV

Plasmid pCMV.BEV.SV40L.BEV(Figure 11) comprises a multiple structural gene unit

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

comprising two BEV polymerase structural genes placed operably and separately under control of the CMV-IE promoter and SV40 late promoter sequences.

5. Plasmid pCMV.BEV.SV40L.VEB

Plasmid pCMV.BEV.SV40L.VEB (Figure 12) comprises a multiple structural gene unit comprising two BEV polymerase structural genes placed operably and separately under control of the CMV-IE promoter and SV40 late promoter sequences. In this plasmid, the BEV polymerase structural gene is expressed in the sense orientation under control of the CMV-IE promoter to produce a translatable mRNA, whilst the BEV polymerase structural gene is also expressed under control of the SV40 promoter to produce an antisense mRNA.

6. Plasmid pCMV.SV40LR.cass

Plasmid pCMV.SV40LR.cass (Figure 13) comprises the synthetic poly(A) site and the SV40 late promoter sequence, sub-cloned such that the CMV-IE and the SV40 late promoters drive transcription, in the opposite direction, of a structural gene or a multiple structural gene unit which is inserted into the multiple cloning site of this plasmid vector. A *Bgl*II site is positioned between the opposing CMV- IE and SV40 late promoter sequences in this plasmid.

7. Plasmid pCMV.BEV.SV40LR

Plasmid pCMV.BEV.SV40LR (Figure 14) comprises a structural gene comprising the entire BEV polymerase open reading frame placed operably and separately under control of opposing CMV-IE promoter and SV40 late promoter sequences, thereby potentially producing BEV polymerase transcripts at least from both strands of the full-length BEV polymerase structural gene. To produce plasmid pCMV.BEV.SV40LR, a translatable BEV polymerase structural gene into the unique *Bgl*II site of plasmid pCMV.SV40LR.cass (Figure 13), such that the BEV open reading frame is present in the sense orientation relative to the CMV-IE promoter sequence.

20-04-2005

Printed: 22-04-2005

TIPA

99910592

P:\OPER\MRO\AG-GENE3.PRV - 15/3/98

C) Genetic constructs comprising the tyrosinase gene or a fragment thereof

Isolation of the tyrosinase open reading frame

The tyrosinase structural gene is isolated by polymerase chain reaction, from mRNA derived from murine cells, using the following oligonucleotide primers under standard polymerase chain reaction conditions:

Tyr 5′(forward primer; SEQ ID NO:5):

5′-CCCGGGGCTTAGTGTAAAACAGGCTGAGAG-3′; and

Tyr 3′ (reverse primer; SEQ ID NO:6):

5′-CCCGGGCAAATCCCAGTCATTTCTTAGAAAC-3′.

Nucleotide residues 1 to 6 in each primer represent a *Sma*I cloning site. Nucleotides 7 to 30 of primer Tyr 5′ correspond to the 5′-end of the murine tyrosinase cDNA sequence disclosed in GenBank Accession No. M20234 (Kwon *et al*, 1988). Nucleotides 7 to 31 of primer Tyr 3′ correspond to the complement of the nucleotide sequence of the 3′-end of the murine tyrosinase cDNA sequence.

Isolation of the OPRSV1 promoter

A DNA fragment comprising the OPRSVI promoter, SV40 intron, *lac* operator sequence, multiple cloning site (MCS) and thymidine kinase (TK) poly(A) sequence was excised from plasmid pOPRSVI/MCS (Stratagene).

1. Plasmid pCMV.TYR

Plasmid pCMV.TYR (Figure 15) comprises the complete mouse tyrosinase cDNA sequence placed operably in connection, in the sense orientation, with the CMV-IE promoter sequence and upstream of the SV40 polyadenylation sequence.

2. Plasmid pCMV.TYRLIB

Plasmid pCMV.TYRLIB (Figure 16) comprises a structural gene or multiple structural gene unit

20-04-2005

which comprises one or more tyrosinase gene fragments of approximately100 to 200 base pairs in length each, placed operably in connection with the CMV-IE promoter sequence and upstream of the SV40 polyadenylation signal. To produce pCMV.TYRLIB, blunt-ended fragments of the tyrosinase gene are produced, for example, by sonication or mechanical shearing and end-repair using T4 DNA polymerase and cloned in operable connection with the CMV IE promoter. Accordingly, the structural gene insert in plasmid pCMV.TYRLIB is variable and an representative library of pCMV.TYRLIB plasmids, covering the complete tyrosinase gene sequence, may be produced using such procedures. The present invention clearly encompasses such representative libraries.

Those skilled in the art will recognise that such procedures are also useful for structural genes other than tyrosinase and, as a consequence, the present invention clearly extends to synthetic genes and genetic constructs wherein the structural gene present in pCMV.TYRLIB is a structural gene other than a tyrosinase gene fragment.

3. Plasmid pCMVLacI.OPRSV1.GFP.TYR

Plasmid pCMVLacI.OPRSV1.GFP.TYR (Figure 17) is a dual construct in which the CMV IE promoter drives expression of the *lac*I gene and the mRNA of the mouse tyrosinase cDNA or a fragment thereof, whilst the OPRSVI promoter drives expression of GFP operably under control of the *lac*I gene. The construct is designed such that the mouse tyrosinase gene is fused to the 3 ' untranslated region of the *lac*I gene via a unique *Bsa*B1 cloning site. This cloning site is located after the stop codon of the *lac*I coding sequence, but before the SV40 polyadenylation signal. The construct also contains the hygromycin-resistance gene as a selection marker.

D) Genetic constructs comprising the *lac*I and green fluorescent protein (GFP) open reading frames

Isolation of the GFP coding region

The GFP open reading frame was amplified from pEGFP-N1 MCS (Clontech) as a *Xho*I-to-*Not*I fragment.

Isolation of the *lac*I coding region

The *Lac*I coding region was isolated from plasmid pCMV.LacI (Stratagene) as a *Hin*dIII- to-*Bsa*B1 fragment.

1. Plasmid pCMVLacI.OPRSV1.GFP.cass

Plasmid pCMVLacI.OPRSVI.GFP.cass (Figure 18) comprises the GFP coding region cloned immediately downstream of the OPRSV1 promoter and the *lac*I structural gene operably under control of the CMV IE promoter sequence. This plasmid is designed such that a structural gene or multiple structural gene unit can be fused to the 3 '-untranslated region of the *lac*I gene, by cloning directly into the unique *Bsa*B1 cloning site which is located after the *lac*I stop codon and before an SV40 polyadenylation signal, thereby placing expression of said structural gene or multiple structural gene operably under control of the CMV IE promoter, such that it is co-expressed with the *lac*I gene.

Alternatively, the *Bsa*B1 site may be modified to facilitate cloning of the structural gene or multiple structural gene unit downstream of the *lac*I gene, for example by the addition of linkers or adaptors.

This construct also contains the antibiotic selectable marker for hygromycin resistance.

D) Genetic constructs comprising the *lac*I open reading frame

1. Plasmid pCMV.Lac

Plasmid pCMV.Lac (Figure 19) contains a CMV IE promoter driving expression of the lac repressor protein encoded by the *Escherichia coli lac*I gene. Accordingly, the open reading frame of the *Lac*I gene is cloned in the sense orientation with respect to the CMV IE promoter sequence in this plasmid. This construct also contains the selectable marker for neomycin resistance.

EXAMPLE 2

Inactivation of virus gene expression in mammals.

Viral immune lines are created by expressing viral sequences in stably transformed cell lines.

In particular, lytic viruses are used for this approach since cell lysis provides very simple screens and also offer the ability to directly select for potentially rare transformation events which might create viral immunity. Sub-genomic fragments derived from a simple single stranded RNA virus (Bovine enterovirus - BEV) or a complex double stranded DNA virus, Herpes Simplex Virus I (HSV I) are cloned into a suitable vector and expressed in transformed cells. Mammalian cell lines are transformed with genetic constructs designed to express viral sequences driven by the strong cytomegalovirus (CMV-IE) promoter. Sequences utilised include specific viral replicase genes. Random "shotgun" libraries comprising repesentative viral gene sequences, may also be used and the introduced disspersed nucleic acid molcule, to target the expression of virus sequences.

Exemplary genetic constructs for use in this procedure, comprising nucleotide sequecnes derived from the BEV RNA-dependent RNA polymerase gene, are presented in Figures 1 to 14.

For viral polymerase constructs, large numbers (approximately 100) of transformed cell lines are generated and infected with the respective virus. For cells transformed with shotgun libraries very large numbers (hundreds) of transformed lines are generated and screened in bulk for viral immunity. Following virus challenge, resistant cell lines are selected and analysed further to determine the sequences conferring immunity thereon.

Resistant cell lines are supportive of the ability of the introduced nucleotide sequences to inactivate viral gene expression in a mammalian system.

Additionally, resistant lines obtained from such experiments are used to more precisely define molecular and biochemical characteristics of the modulation which is observed.

Printed: 22-04-2005

TIPAT

9991 0592

P:\OPER\MRO\AG-GENE3.PRV - 19/3/98

EXAMPLE 3

Inactivation of pigment biosynthesis in transgenic mice.

To investigate repression of gene expression in transgenic animals using the presently described invention, genetic constructs are produced to target expression of one or more pigment biosynthetic gene in transgenic mice. Pigment production in mice is well characterised genetically (Jackson, 1995). By targeting expression of the tyrosinase gene in transgenic mice, pigment production is inhibited. A simple visual assay for the efficacy of the invention measures the occurrence of albinism in genetically-black mice. Furthermore, since melanocytes can be readily cultured from mature animals this system offers the ability to undertake molecular analysis of gene inactivation events.

Constructs comprising a promoter such as the CMV promoter driving tyrosinase cDNA constructs are prepared and used to micro-inject mouse embryos.

Random shotgun libraries comprising representative nucleotide sequences of up to approximately 300bp in length each, collectively covering the entire tyrosinase gene sequence, may also be used to target the expression of the tyrosinase gene. This approach allows for selection of those nucleotide sequences which provide for optimum inactivation of tyrosinase gene expression. The random shotgun library may also be micro-injected into mouse embryos.

Exemplary genetic constructs for use in this procedure, targeting expression of the murine tyrosinase gene, are presented in Figures 15 to 17.

Gene inactivation events are monitored visually. Sectored gene inactivation events are easily detected by the occurrence of mottled or patchy colouration in transgenic animals. Animals showing evidence of extreme albinism or strong sectored albinism are selected. Melanocytes are cultured from such lines and subjected to more detailed analyses.

20-04-2005

Printed: 22-04-2005 TIPA 99910592

EXAMPLE 4

Inactivation of expression of nuclear genes using a simple visual reporter system.

To create a simple visual reporter system, cell lines are first stably transformed with marker constructs consisting of two genes, wherein the first of said genes encodes a trans-regulatory protein, for example the lac repressor which is normaliy capable of repressing the expression of the *Escherichia coli lacZ* gene and wherein the second gene corresponds to a visual marker gene, for example the green fluorescent protein (GFP) or tyrosinase gene placed operably in connection with a promoter comprising operator sequences for binding of said repressor.

To detect repression of expression of the reporter gene, the expression of the repressor gene is targeted by introducing a further genetic construct comprising a third nucleic acid molecule which is capable of repressing, reducing or repressing expression of said first gene. As a consequence, reporter gene expression is induced in cells which do not express the trans-regulatory protein, permitting visual assay of the efficacy of the introduced nucleic acid molecule.

Cell lines are transformed with the marker construct. Cloned lines are selected which show little or no background expression of the reporter gene, but high levels of expression when induced by IPTG (for lac). Once characterised lines are established these are super-transformed with constructs expressing the third nucleic acid molecule which targets expression of the trans-regulatory repressor protein. Repression of expression of the first nucleic acid molecule is then monitored visually by measuring reporter gene expression levels. Cell lines in which such repression of gene expression is identified are purified for detailed analysis.

Cell lines with such easily scored markers also provide ideal systems for examining the effects of transient delivery of constructs as either gene cassettes, by using viral delivery systems or by direct delivery of one or more oligonucleotides or oligoribonucleotides.

420 20-04-2005

Printed: 22-04-2005 TIRA1 99910592

EXAMPLE 5

Transcription-mediated inactivation of *lac*I gene expression in mammalian cells

Background

In the presence of lactose as the sole carbon source, the *E. coli lac* operon expresses large quantities of the enzyme β-galactosidase, which is encoded by the *lac*Z. The three polycistronic genes of the *lac* operon are under transcriptional control of the *lac* promoter-operator sequence, which in turn is under the negative control of the *lac* repressor; a DNA binding protein product of the *lac*I gene. In the presence of one of a variety of beta-galactosides, the affinity of the repressor protein for the lac promoter-operator sequence is lowered, allowing RNA polymerase to bind to the promoter region of the genes resulting in transcription of the *lac* operon.

To produce transcription-mediated inactivation of *lac*I gene expression in mammalian cells, the present inventors have utilised the transcriptional repression function of the *lac*I repressor gene as the target gene and a visual reporter gene to detect successful inactivation of gene expression.

Method

Two genetic constructs are produced as follows:

1. A first genetic construct is produced which contains two promoters, wherein the first promoter, for example CMV IE, drives expression of the *lac*I gene and the second promoter comprising *lac* operator sequences, for example the OPRSV1 promoter (a modified RSV-LTR promoter derived from the vector pOPRSV1/MCS [Stratagene]) drives expression of a visual reporter gene such as the GFP reporter gene. In such a system, when the *lac* repressor protein binds to the *lac* operator sequences, transcription of the visual reporter gene is inhibited. In the presence of IPTG, the *lac*I repressor no longer binds the *lac* operator sequence and the second promoter is capable of regulating transcription of the visual reporter gene. Accordingly, a positive phenotype (in particular visual reporter gene expression) may be monitored in transformed cells.

20-04-2005

2. A second genetic construct is produced which comprises a third promoter sequence, for example the CMV IE promoter, driving expression of the *lac*I gene.

It is particularly preferred that the first and second genetic constructs contains different selectable marker genes to facilitate their co-selection in transformed cells, for example the genes encoding neomycin resistance and hygromycin resistance, amongst others.

In this system, the *lac*I gene of the first genetic construct comprises the target gene, whilst the *lac*I gene of the second genetic construct comprises the introduced dispersed or foreign nucleic acid molecule.

An exemplary genetic construct, suitable for use as the first genetic construct which is referred to herein, comprises the CMV IE promoter driving *lac*I gene expression and the OPRSV1 promoter driving expression of the GFP gene, as set forth in Figure 18. An exemplary genetic construct, suitable for use as the second genetic construct which is referred to herein, comprises the CMV IE promoter driving *lac*I gene expression, as set forth in Figure 19.

The first genetic construct is transformed into a suitable mammalian cell line, for example MDBK cells using standard techniques and transformed cell lines are selected which are able to derepress the *lac* operator, as determined by increased expression of the visual reporter gene when IPTG is added to the cell culture media.

Stable lines comprising the first genetic construct and which reproducibly derepress the *lac* operator in the presence of IPTG are 'supertransformed' with the second genetic construct. Cell lines which contain both genetic constructs are selected by their ability to grow on different selective media.

On the other hand, cell lines comprising only the second genetic construct are able to grow on only one selective medium. Such cells preferably produce translatable or non-translatable *lac*I mRNA constitutively, by virtue of the promoter used to drive *lac*I gene expression therein.

Printed: 22-04-2005 TIPA1 9991059

P:\OPER\MROAG-GENE3.PRV - 19/3/98

In the selected cell lines comprising both genetic constructs, inactivation of the *lac*I target gene of the first genetic construct and/or selective degradation of the *lac*I repressor mRNA by the presence or expression of the introduced dispersed or foreign nucleic acid molecule (i.e. second genetic construct) may be monitored by determining visual reporter gene expression levels (Table 1). In the absence or presence of IPTG, cells comprising both the first and second genetic construct fail to produce the *lac* repressor protein, the second promoter is derepressed constitutively and expression of the reporter gene does not require the presence of IPTG in the media.

TABLE 1

GENETIC CONSTRUCT INTRODUCED	RELATIVE REPORTER GENE EXPRESSION	
	No IPTG in media	Plus IPTG in media
Construct I only	-	+++
Construct II only	-	-
Constructs I and II	+++	+++

EXAMPLE 6

Transcription-mediated inactivation of endogenous gene expression in mammalian cells

To investigate transcriptional mediated inactivation of expression of cellular genes in a mammalian system, the present inventors have utilised the *lac*I repressor gene as an ancillary target gene and a visual reporter gene to detect successful inactivation of gene expression in Madin Darby Bovine Kidney (MDBK) cells. The primary target gene in these experiments is a cellular or endogenous gene, such as the murine tyrosinase gene.

Two genetic constructs are produced as follows:

1. A first genetic construct is produced which contains two promoters, wherein the

20-04-2005

first promoter, for example CMV IE, drives expression of both the *lac*I gene and the fused endogenous gene of interest, and the second promoter comprising *lac* operator sequences, for example the OPRSV1 promoter (a modified RSV-LTR promoter derived from the vector pOPRSV1/MCS [Stratagene]) drives expression of a visual reporter gene such as the GFP reporter gene.

2. A second genetic construct is produced which comprises a third promoter sequence, for example the CMV IE promoter, driving expression of two fused structural genes, wherein the first structural gene comprises the *lac*I gene and the second structural gene comprises the endogenous gene of interest placed between said first structural gene and a transcription termination sequence.

It is particularly preferred that the first and second genetic constructs contains different selectable marker genes to facilitate their co-selection in transformed cells, for example the genes encoding neomycin resistance and hygromycin resistance, amongst others.

In this system, the *lac*I gene of the first genetic construct comprises the ancillary target gene and the endogenous gene of the first genetic construct comprises the primary target gene, whilst the *lac*I structural gene and endogenous structural gene of the second genetic construct comprise the introduced dispersed or foreign nucleic acid molecule.

An exemplary genetic construct, suitable for use as the first genetic construct which is referred to in this Example, comprises the CMV IE promoter driving *lac*I and murine tyrosinase gene expression and the OPRSV1 promoter driving expression of the GFP gene, as set forth in Figure 17. Exemplary genetic constructs, suitable for use as the second genetic construct which is referred to herein, comprise the CMV IE promoter driving *lac*I gene and murine tyrosinase gene expression. In this context, the murine tyrosinase-specific nucleic acid molecule may comprise the full gene sequence or a fragment thereof, such as a gene fragment which has been cloned into a random shotgun library of representative tyrosinase gene fragments.

Printed: 22-04-2005 TIPAT 99910592

In use, the first genetic construct is transformed into a suitable mammalian cell line, for example MDBK cells using standard techniques and transformed cell lines are selected which are able to derepress the *lac* operator, as determined by increased expression of the visual reporter gene when IPTG is added to the cell culture media.

Stable lines comprising the first genetic construct and which reproducibly derepress the *lac* operator in the presence of IPTG are 'supertransformed' with the second genetic construct. Cell lines which contain both genetic constructs are selected by their ability to grow on different selective media. On the other hand, cell lines comprising only the first or second genetic construct are able to grow on only one selective medium.

In cells transformed with the first genetic construct alone, mRNA encoding the *lacI* repressor protein and the endogenous gene of interest is produced as a single transcript, wherein at least the *lacI* mRNA is further capable of being translated. In such cells, when the *lacI* mRNA is translated to produce a functional *lac* repressor protein, the *lac* repressor protein binds to the *lac* operator sequences present in the second promoter, such that transcription of the visual reporter gene is inhibited. Accordingly, in such circumstances, transcription of the endogenous gene is linked to the absence of detectable reporter gene expression. In the presence of IPTG, the *lacI* repressor no longer binds the *lac* operator sequence and the second promoter is capable of regulating transcription of the visual reporter gene, however reporter gene expression may be independent of transcription of the endogenous gene. Accordingly, a positive phenotype (in particular visual reporter gene expression) may be monitored in transformed cells only in the absence of IPTG.

In cells transformed with the second genetic construct alone, there is no visual reporter gene and, as a consequence, expression of the visual reporter gene will not be detected. However, mRNA encoding the endogenous gene may be detected in such cells, irrespective of whether IPTG is added to the culture medium.

In cells transformed with both the first and second genetic constructs, expression of both the

425 20-04-2005

visual reporter gene and the endogenous gene is independent of IPTG concentration in the culture medium, wherein expression of the visual reporter gene is detectable both in the presence and absence of IPTG and expression of the endogenous gene is undetectable both in the presence and absence of IPTG (Table 2), suggesting that inactivation of both the ancillary and primary target genes has occurred. Absent inactivation, it would be expected that a significant proportion of cells would express the endogenous gene at a detectable level and fail to express the visual reporter gene.

TABLE 2

GENETIC CONSTRUCT INTRODUCED	RELATIVE GENE EXPRESSION			
	VISUAL REPORTER GENE		ENDOGENOUS GENE	
	No IPTG	Plus IPTG	No IPTG	Plus IPTG
Construct I only	-	+++	+++	+++
Construct II only	-	-	+++	+++
Construct I and II	+++	+++	-	-

Printed 22-04-2005 TIPA1 99910592

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: AgGene Australia Pty.Ltd and The Government of Queensland as represented by Queensland Department of Primary Industries

ii) TITLE OF INVENTION: Synthetic genes and genetic constructs comprising same I

iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: DAVIES COLLISON CAVE
(B) STREET: 1 LITTLE COLLINS STREET
(C) CITY: MELBOURNE
(D) STATE: VICTORIA
(E) COUNTRY: AUSTRALIA
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:AU provisional
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: HUGHES EL, JOHN E L

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +61 3 9254 2777
(B) TELEFAX: +61 3 9254 2770
(C) TELEX: AA 31787

4237 20-04-2005

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGCAGATCT AACAATGGCA GGACAAATCG AGTACATC 38

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCGGGATCC TCGAAAGAAT CGTACCACTT C 31

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

Printed: 22-04-2005

TIPAT

99910592

F:\OPER\MRO\AG-GENE3.PRV - 19/3/98

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGCGGATCC TTAGAAAGAA TCGTACCAC 29

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGCAGATCT GGACAAATCG AGTACATC 28

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCCGGGGCTT AGTGTAAAAC AGGCTGAGAG 30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20-04-2005

Printed: 22-04-2005 TIPA1 99910592

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

5 CCCGGGCAAA TCCCAGTCAT TTCTTAGAAA C 31

430 20-04-2005

Printed: 22-04-2005 TIPA 99910592

EQUIVALENTS

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

REFERENCES

1. Dorer, D.R., and Henikoff, S. (1994) *Cell* 7: 993-1002.

2. Jackson, I.J. (1995) *Ann. Rev. Genet.* 28: 189-217.

3. Kwon, B.S. *et al.* (1988) *Biochem. Biophys. Res. Comm.* 153:1301- 1309.

4. Pal-Bhadra, M. *et al.* (1997) *Cell* 90: 479-490.

5. Prasher, D.C. *et al.* (1992) *Gene* 111: 229-233.

DATED this 19TH day of MARCH, 1998

Ag-Gene Australia Ltd AND

State of Queensland through its Department of Primary Industries

by DAVIES COLLISON CAVE

Patent Attorneys for the Applicants

20-04-2005

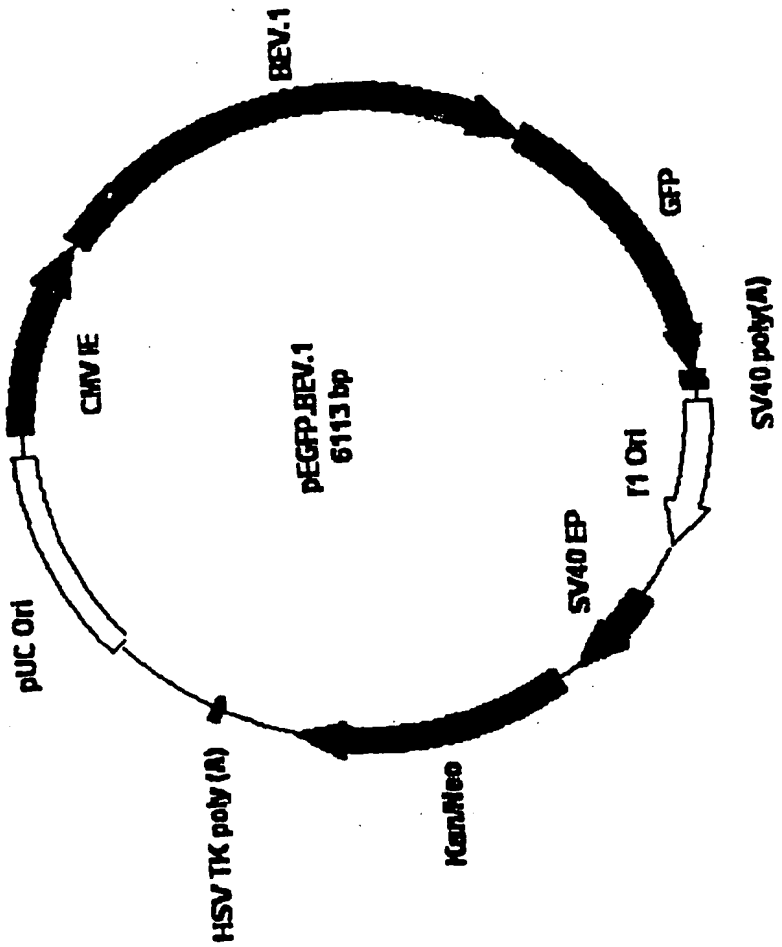

BEV.1
GFP
SV40 poly(A)
f1 Ori
SV40 EP
KanNeo
HSV TK poly (A)
pUC Ori
CMV IE
pEGFP.BEV.1
6113 bp

FIGURE 1

Printed: 28-04-2005

99910592

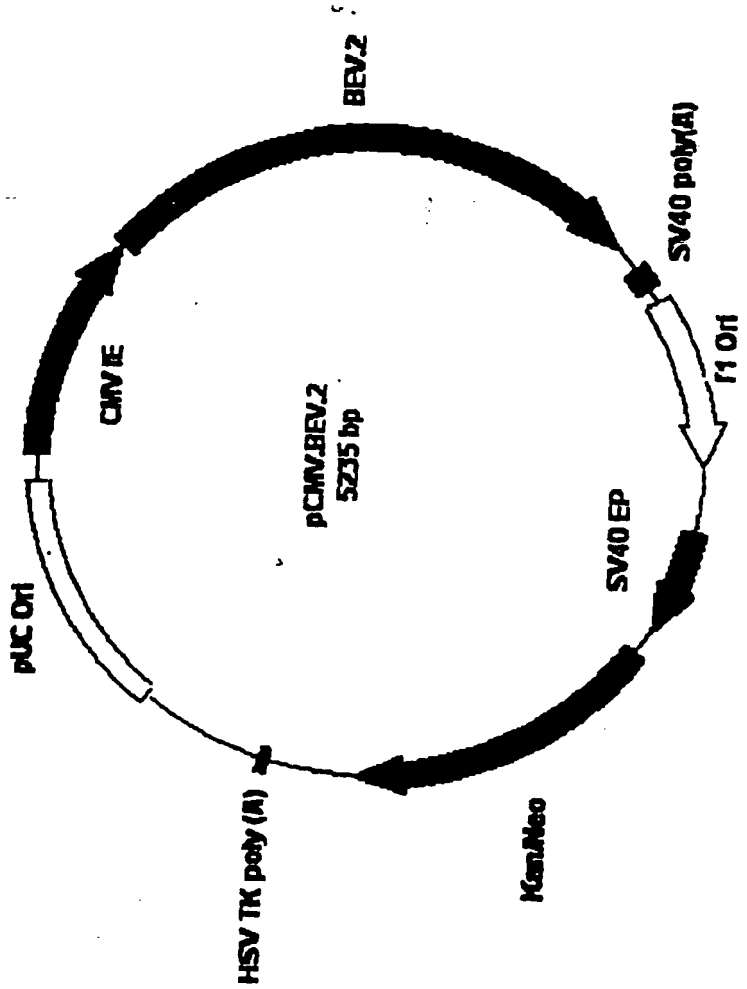


FIGURE 2

20-04-2005

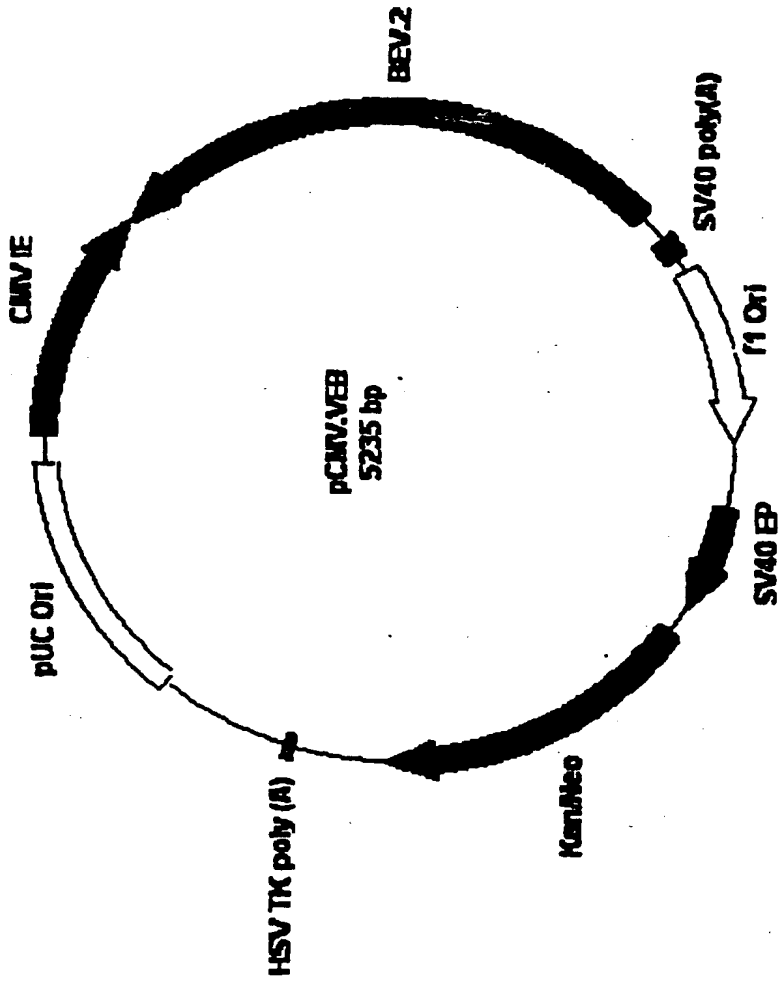

BEV2
SV40 poly(A)
f1 Ori
SV40 EP
KanNeo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.VEB
5235 bp

FIGURE 3

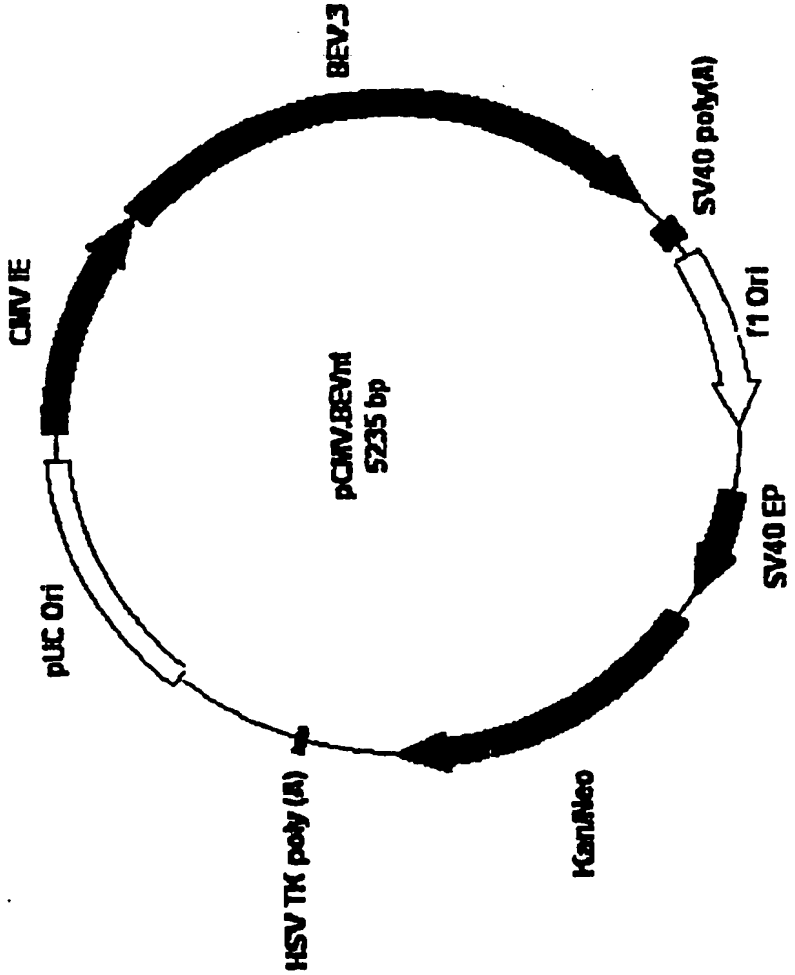

BEV3
SV40 poly(A)
f1 Ori
SV40 EP
Kan/Neo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.BEVnt
5235 bp

FIGURE 4

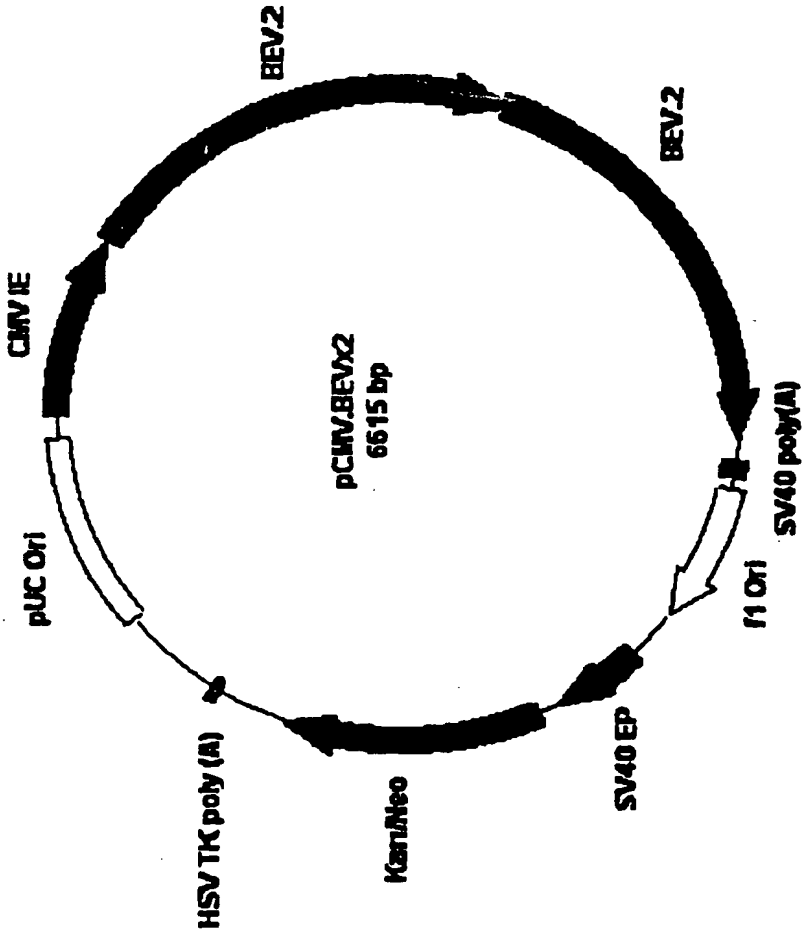

BEV2
BEV2
CMV IE
pCMV.BEVx2
6615 bp
SV40 poly(A)
pUC Ori
f1 Ori
HSV TK poly (A)
KanNeo
SV40 EP

FIGURE 5

Printed: 22-04-2005
TIPA
99910592

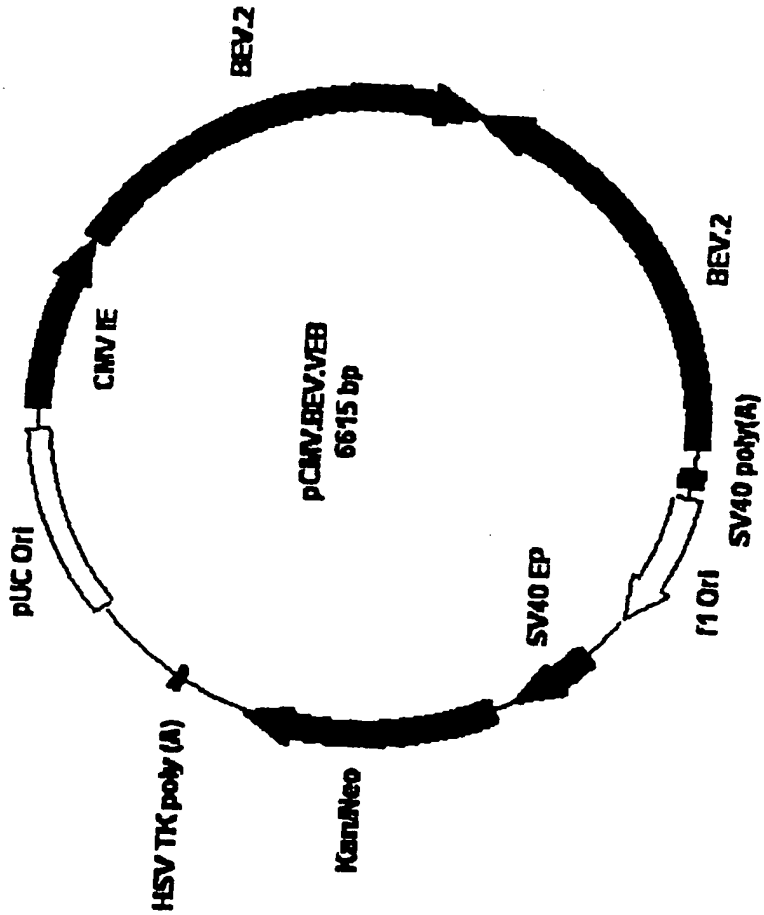


FIGURE 6

20-04-2005

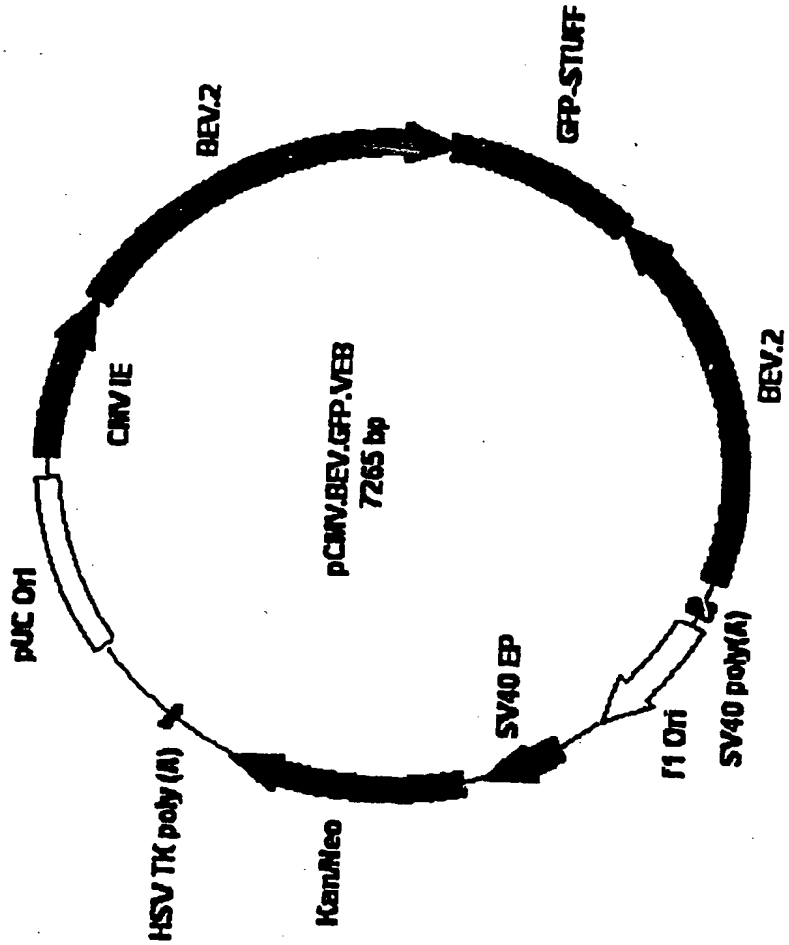

BEV.2
GFP-STUFF
CMV IE
pCMV.BEV.GFP.VEB
7265 bp
BEV.2
pUC Ori
SV40 EP
SV40 poly(A)
f1 Ori
HSV TK poly (A)
KanNeo

FIGURE 7

Printed: 22-04-2005

TIPA

99910592

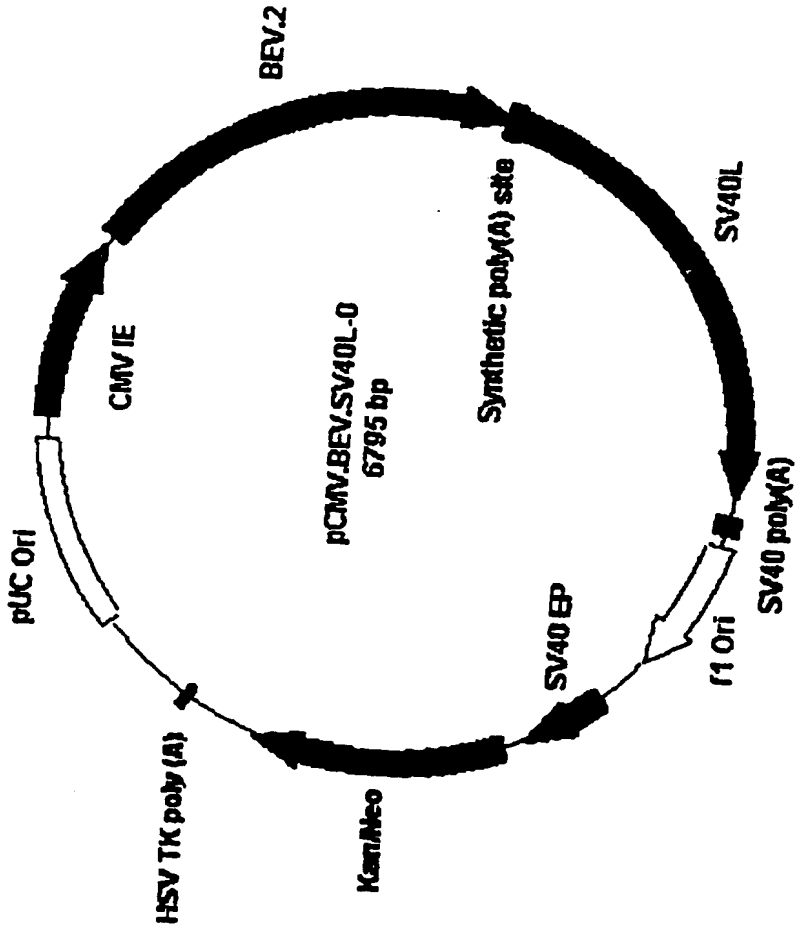


FIGURE 8

RIS

20-04-2005

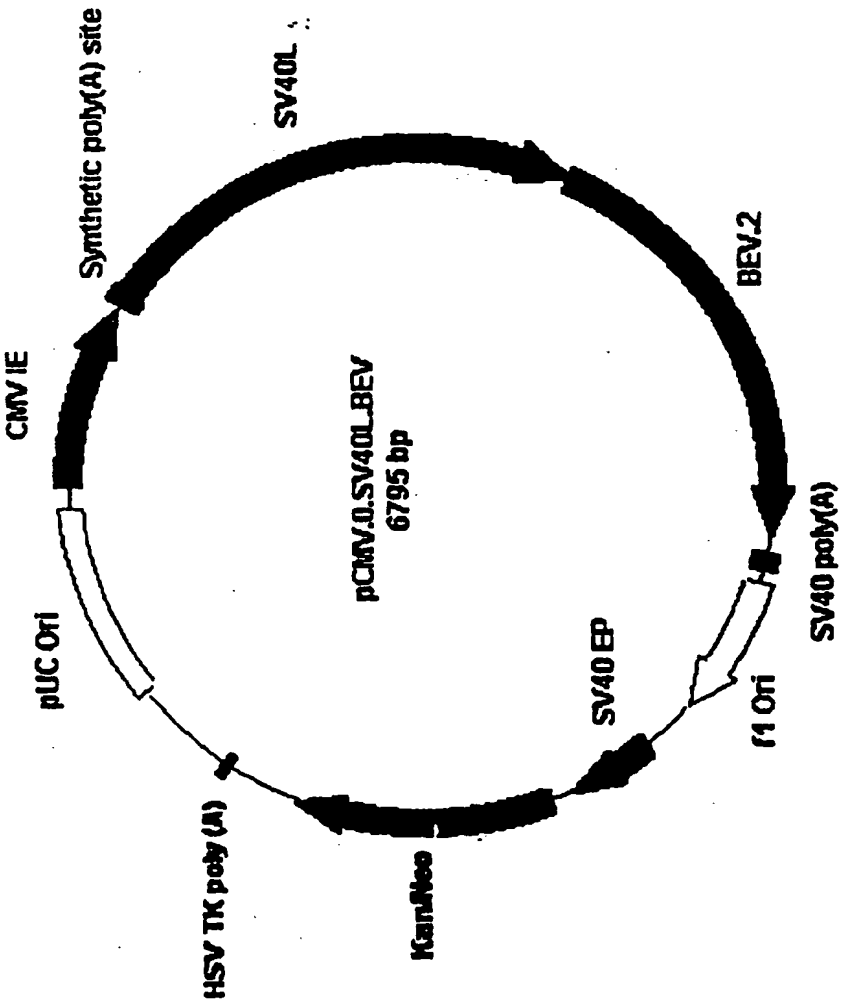

Synthetic poly(A) site
SV40L
BEV.2
CMV IE
pCMV.0.SV40L.BEV
6795 bp
SV40 poly(A)
pUC Ori
SV40 EP
f1 Ori
HSV TK poly (A)
KanRes

FIGURE 9

Printed: 22-04-2005

TIPA1

99910592

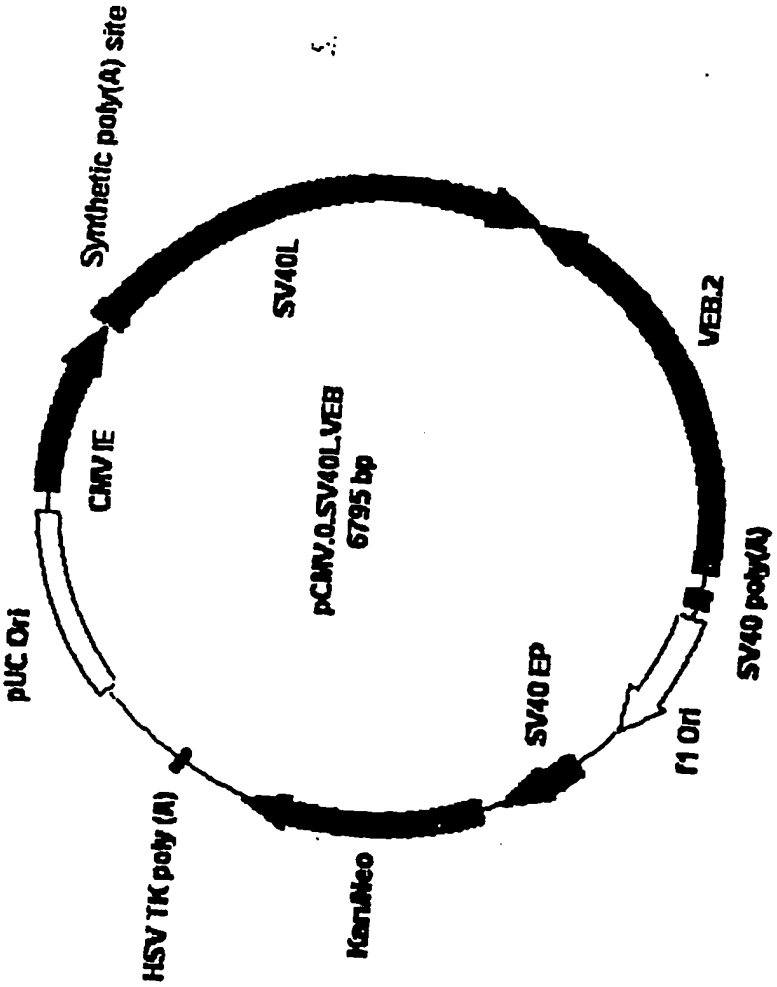


FIGURE 10

20-04-2005

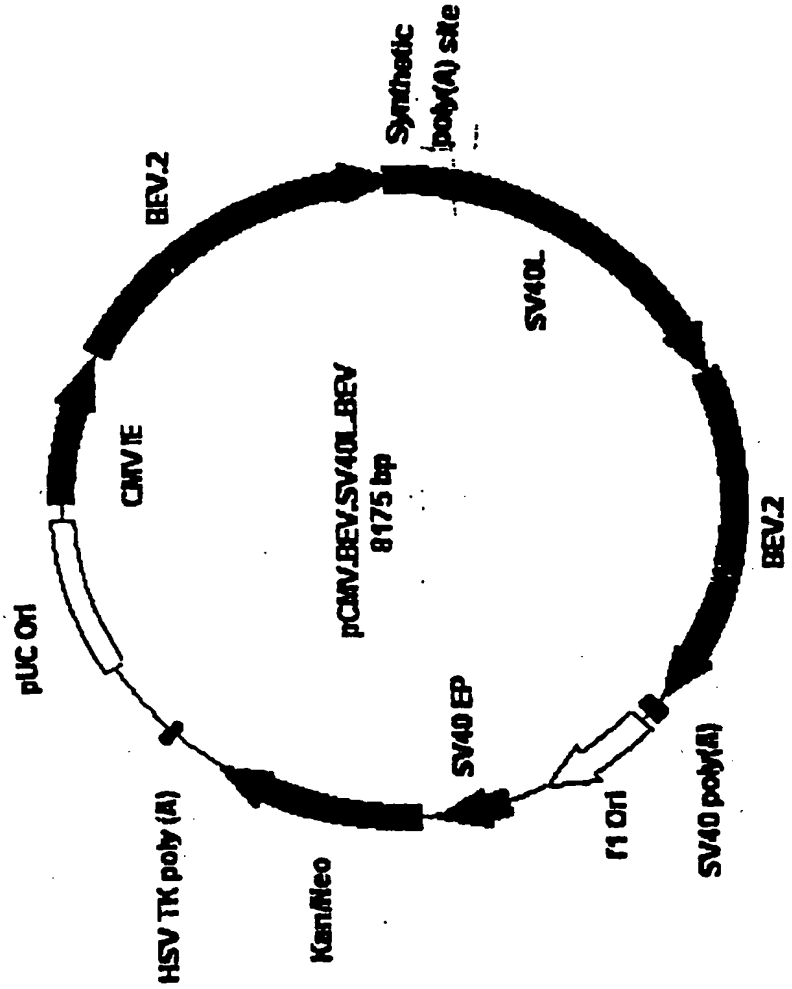

Synthetic poly(A) site
SV40L
BEV.2
f1 Ori
SV40 poly(A)
SV40 EP
KanNeo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.BEV.SV40L.BEV
8175 bp

FIGURE 11

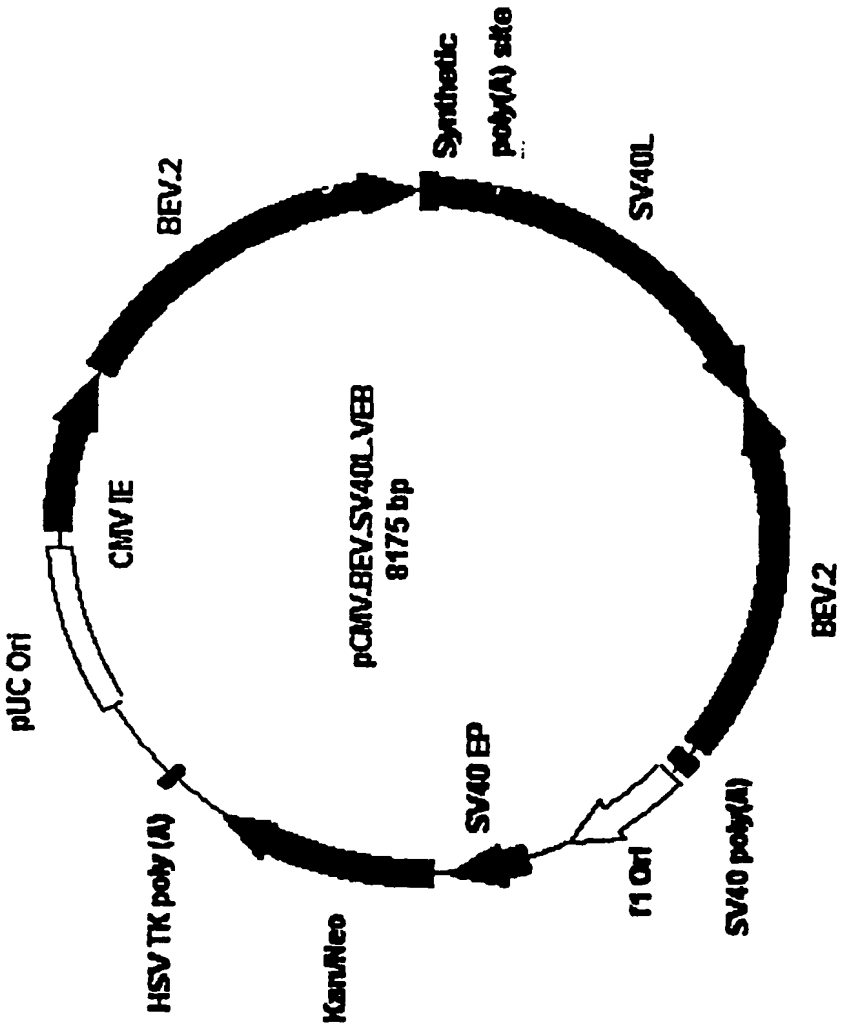

Synthetic poly(A) site
SV40L
BEV2
pCMV.BEV.SV40L.VEB
8175 bp
BEV2
CMV IE
pUC Ori
SV40 EP
SV40 poly(A)
f1 Ori
HSV TK poly (A)
KanuNeo

FIGURE 12

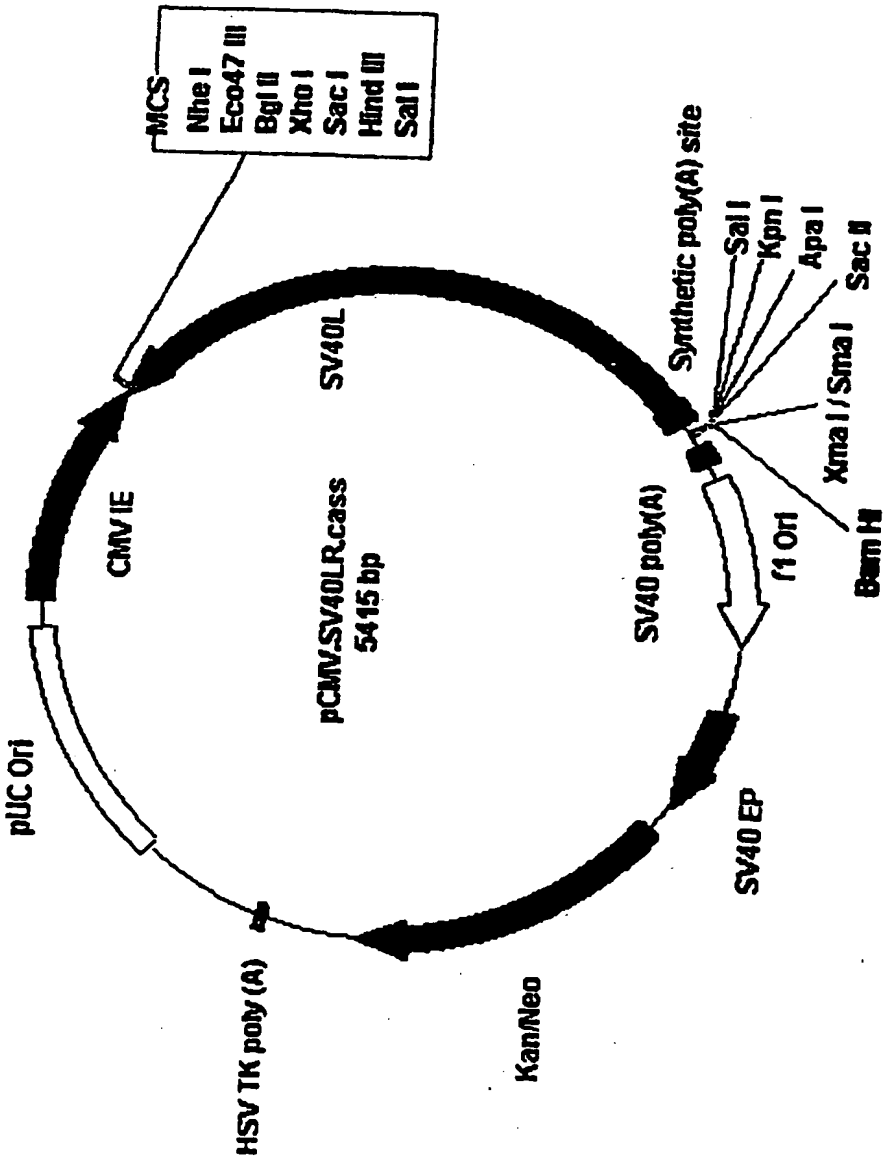

MCS
Nhe I
Eco47 III
Bgl II
Xho I
Sac I
Hind III
Sal I
SV40L
Synthetic poly(A) site
Sal I
Kpn I
Apa I
Sac II
Xma I / Sma I
Bam HI
f1 Ori
SV40 poly(A)
CMV IE
pCMVSV40LRcass
5415 bp
pUC Ori
SV40 EP
HSV TK poly (A)
Kan/Neo

FIGURE 13

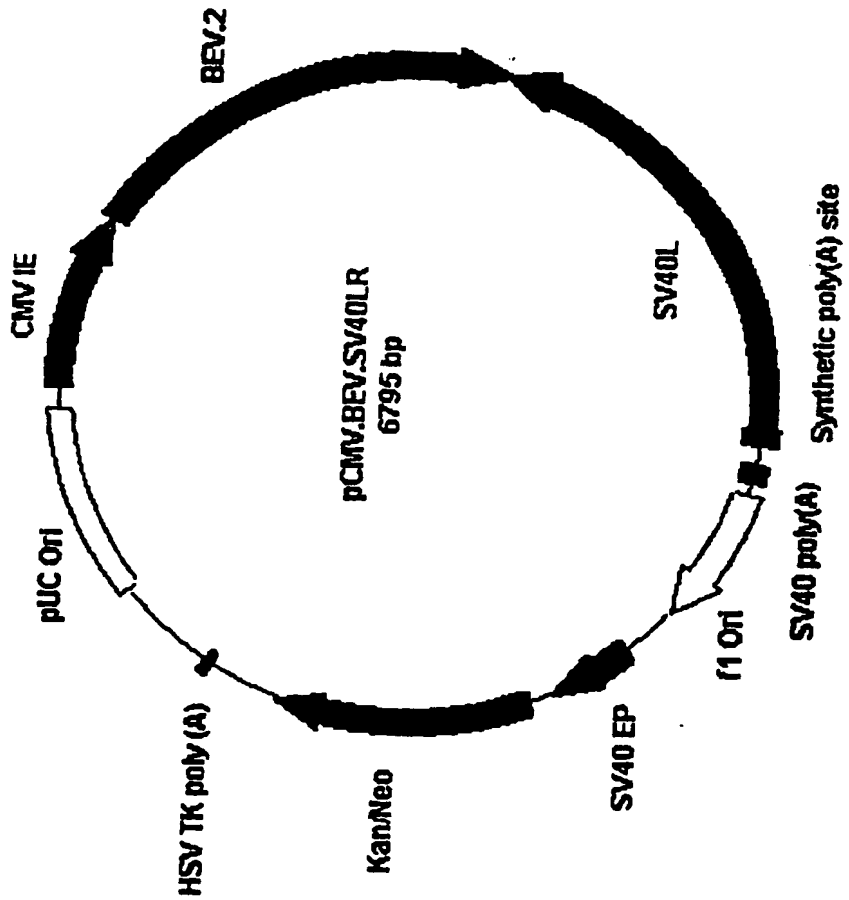

BEV.2
CMV IE
pCMV.BEV.SV40LR
6795 bp
SV40L
Synthetic poly(A) site
pUC Ori
SV40 poly(A)
f1 Ori
HSV TK poly (A)
KanNeo
SV40 EP

FIGURE 14

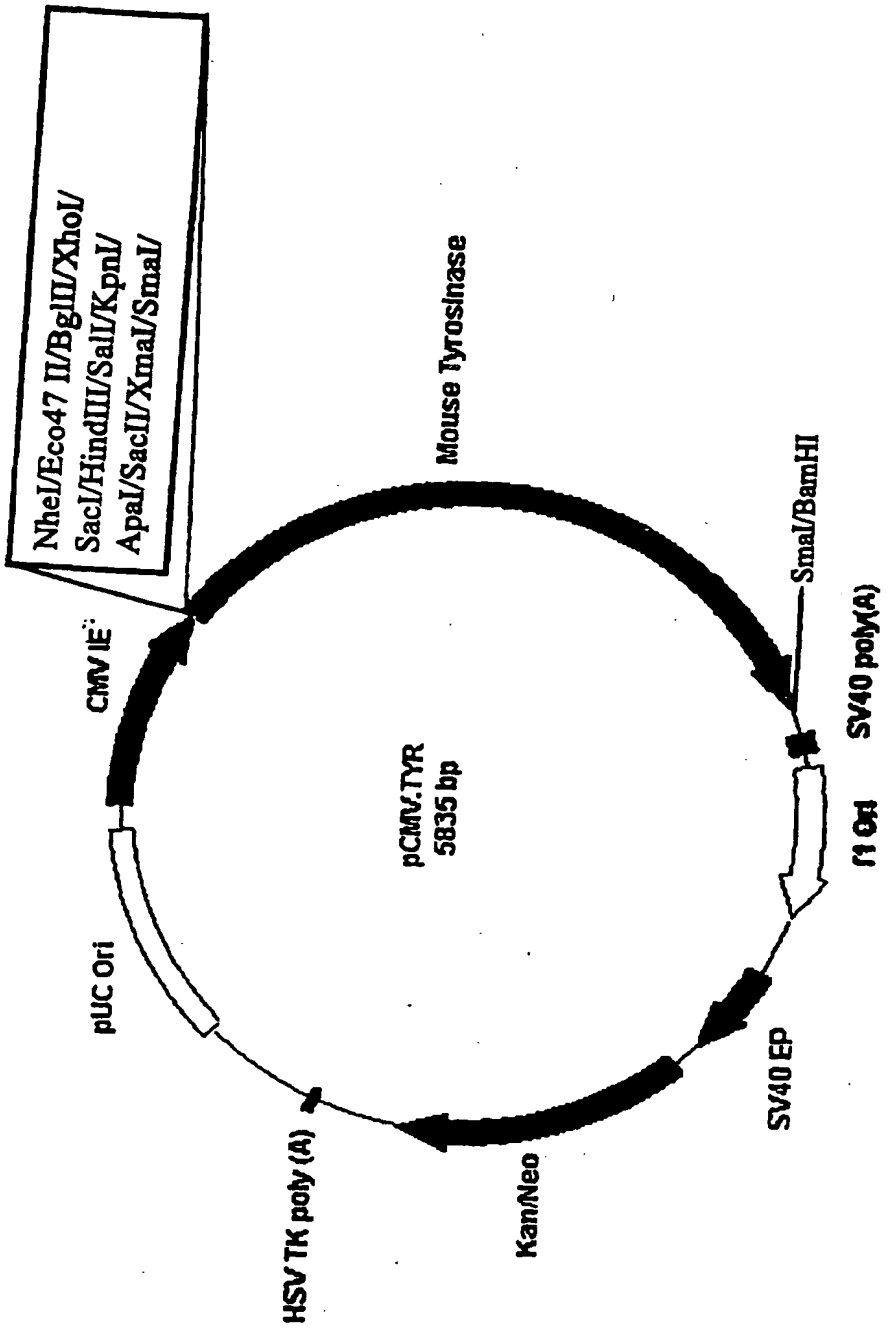

NheI/Eco47 II/BglII/XhoI/
SacI/HindIII/SalI/KpnI/
ApaI/SacII/XmaI/SmaI/
Mouse Tyrosinase
SmaI/BamHI
SV40 poly(A)
f1 Ori
SV40 EP
Kan/Neo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.TYR
5835 bp

FIGURE 15

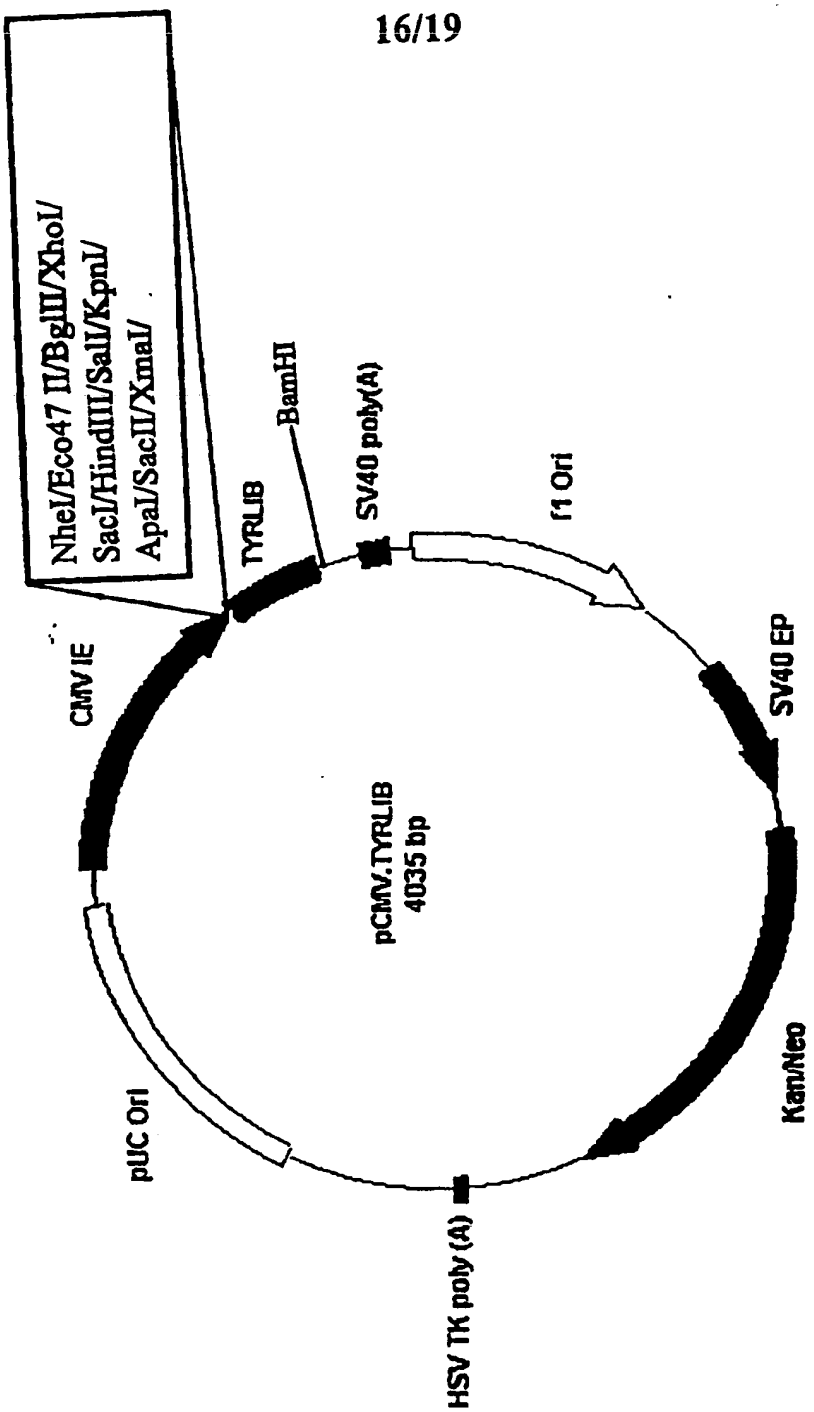

NheI/Eco47 II/BglII/XhoI/
SacI/HindIII/SalI/KpnI/
ApaI/SacII/XmaI/
BamHI
TYRLIB
SV40 poly(A)
f1 Ori
SV40 EP
Kan/Neo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.TYRLIB
4035 bp

FIGURE 16

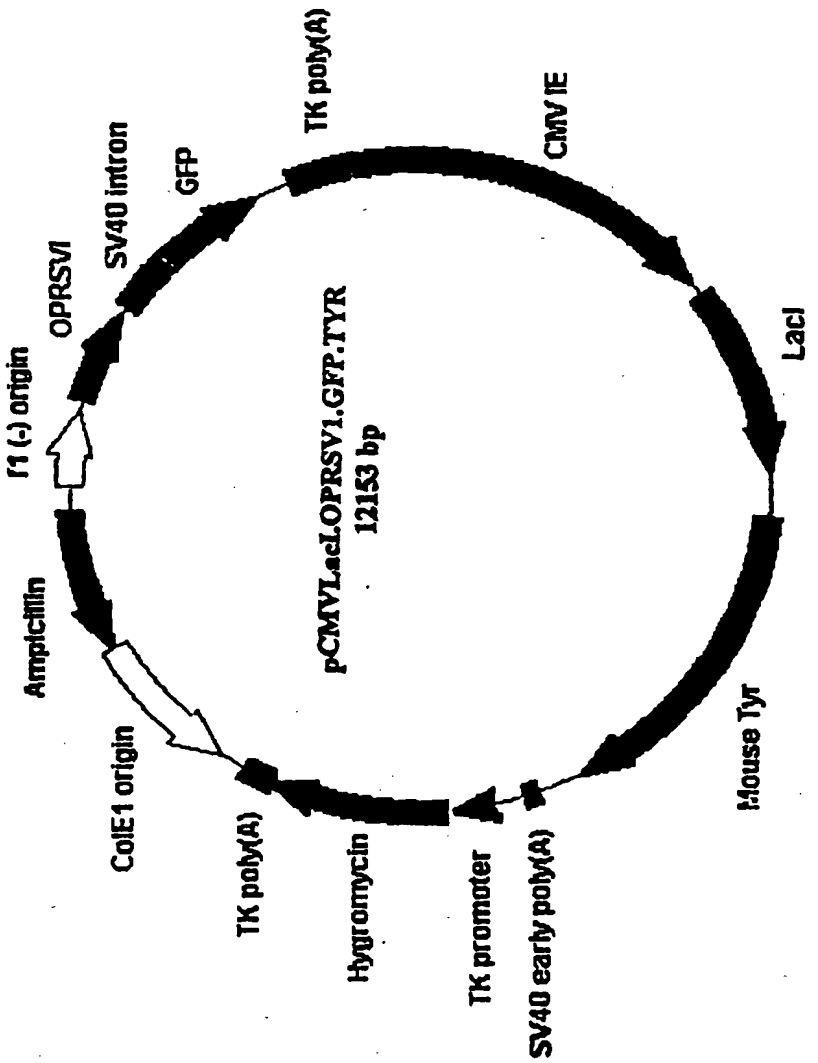

pCMVLacI.OPRSV1.GFP.TYR
12153 bp
TK poly(A)
CMV IE
LacI
Mouse Tyr
SV40 early poly(A)
TK promoter
Hygromycin
TK poly(A)
ColE1 origin
Ampicillin
f1 (-) origin
OPRSVI
SV40 intron
GFP

FIGURE 17

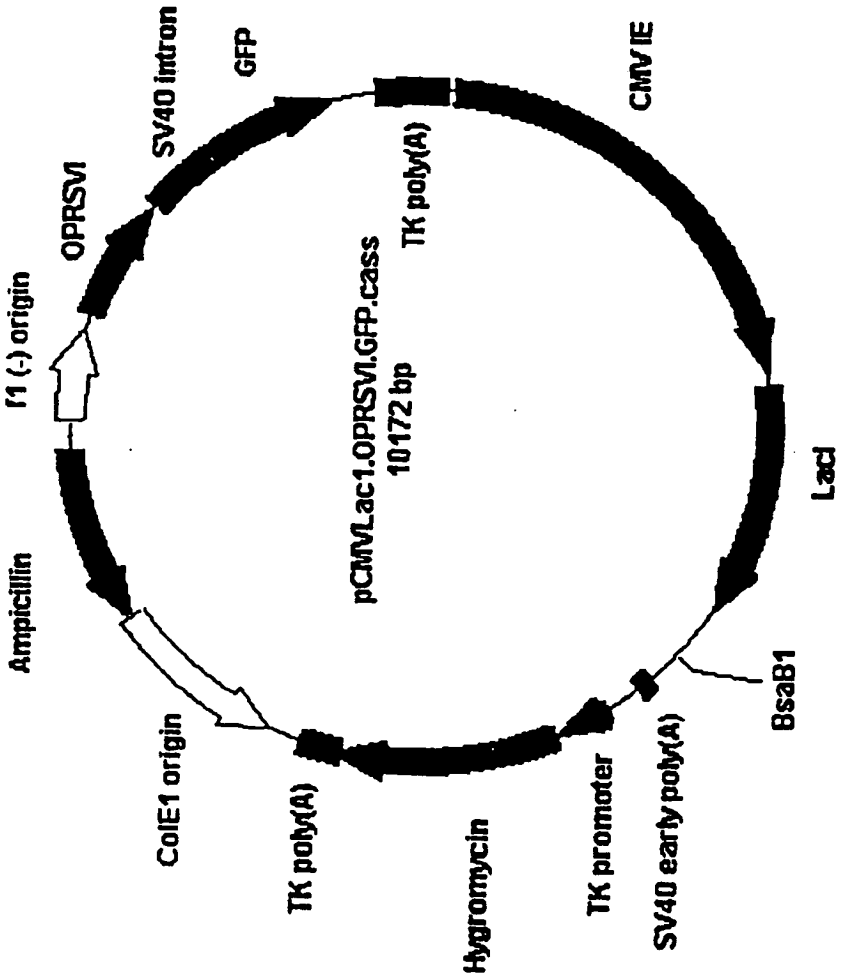

CMV IE
TK poly(A)
GFP
SV40 intron
OPRSVI
f1 (-) origin
Ampicillin
ColE1 origin
TK poly(A)
Hygromycin
TK promoter
SV40 early poly(A)
BsaB1
LacI
pCMVLac1.OPRSVI.GFP.cass
10172 bp

FIGURE 18

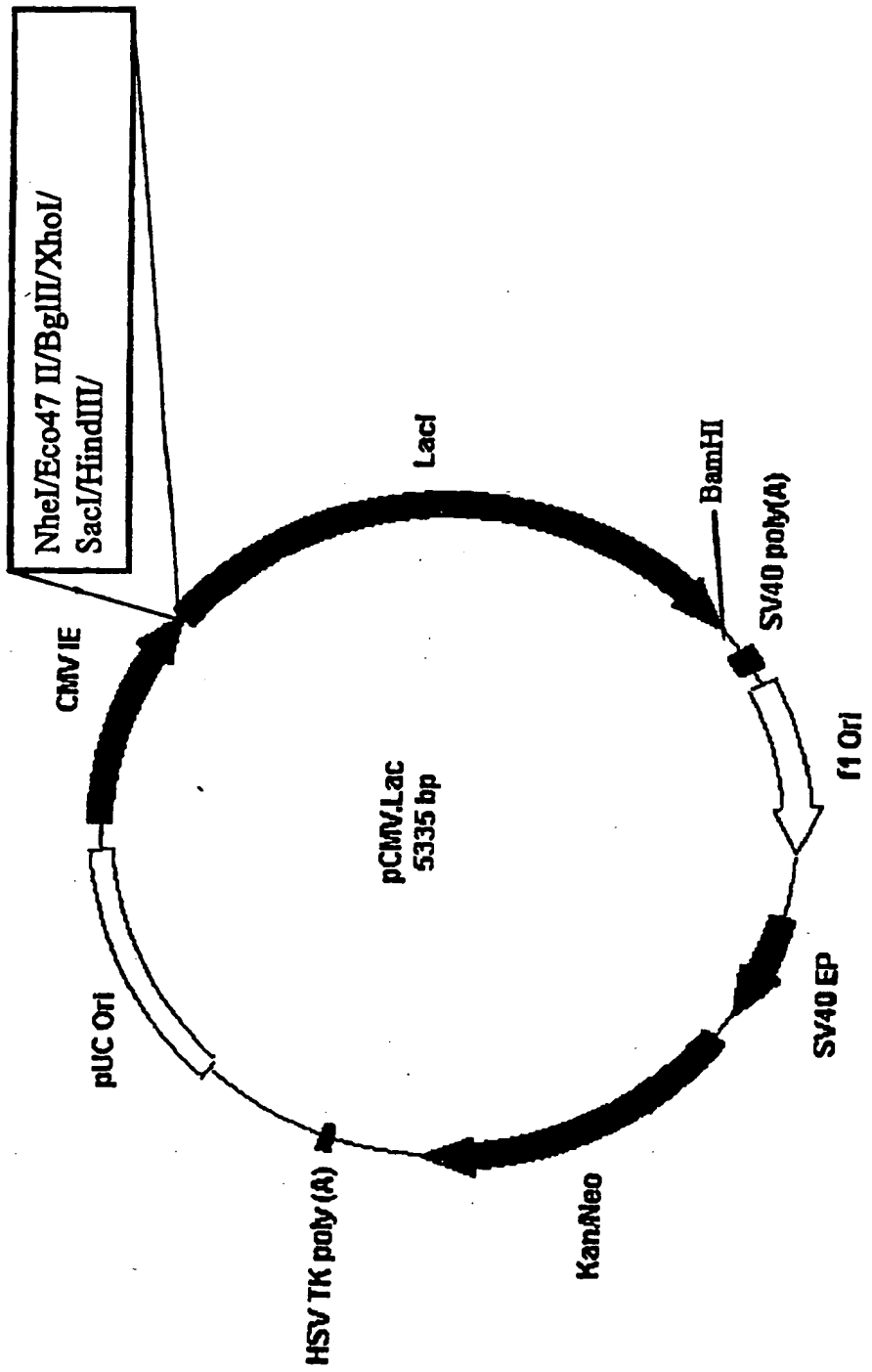

NheI/Eco47 II/BglII/XhoI/
SacI/HindIII/
LacI
BamHI
SV40 poly(A)
f1 Ori
SV40 EP
KanNeo
HSV TK poly (A)
pUC Ori
CMV IE
pCMV.Lac
5335 bp

FIGURE 19

Printed: 22-04-2005 TIPA1 99910592

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/63, 15/82 — A1

(11) International Publication Number: WO 98/53083

(43) International Publication Date: 26 November 1998 (26.11.98)

(21) International Application Number: PCT/GB98/01450

(22) International Filing Date: 20 May 1998 (20.05.98)

(30) Priority Data:
9710475.6 21 May 1997 (21.05.97) GB

(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: GRIERSON, Donald [GB/GB]; University of Nottingham, Sutton Bonnington Campus, Loughborough LE12 5RD (GB). LOWE, Alexandra, Louise [GB/GB]; University of Nottingham, Sutton Bonnington Campus, Loughborough LE12 5RD (GB). HAMILTON, Andrew, John [GB/GB]; University of Nottingham, Sutton Bonnington Campus, Loughborough LE12 5RD (GB).

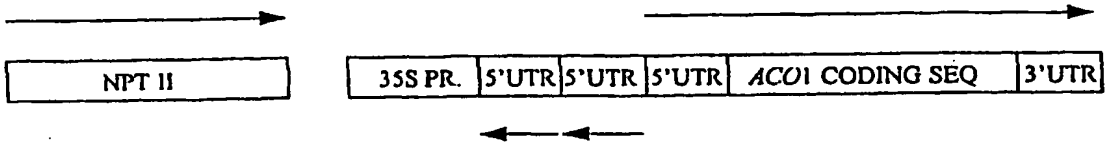
(74) Agents: HUSKISSON, Frank, Mackie et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

EPO - DG 1
20. 04. 2005
(93)

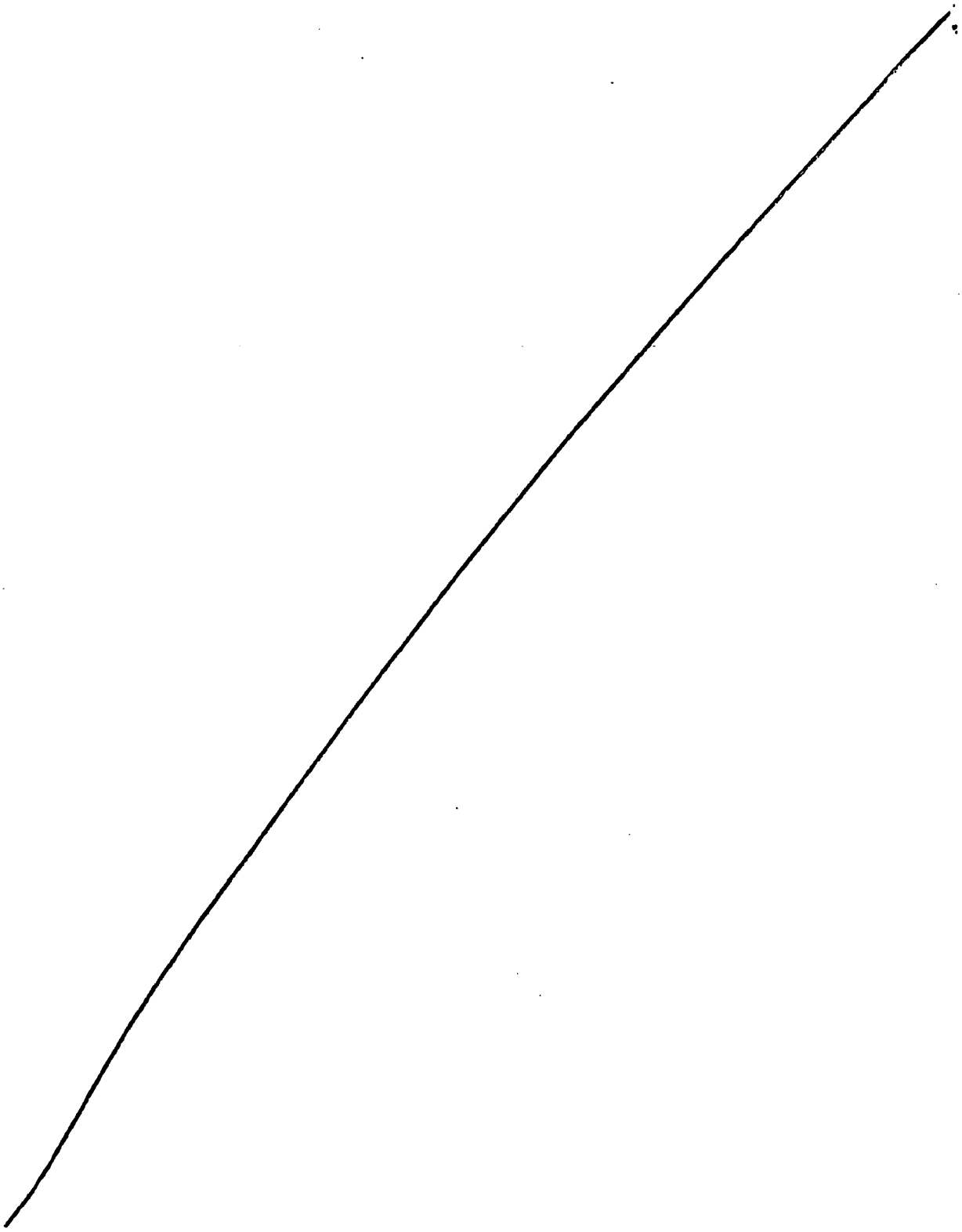
(54) Title: GENE SILENCING



(57) Abstract

Constructs and methods for enhancing the inhibition of a target gene within an organism involve inserting into the gene silencing vector an inverted repeat sequence of all or part of a polynucleotide region within the vector. The inverted repeat sequence may be a synthetic polynucleotide sequence or comprise a modified natural polynucleotide sequence.

4/52 20-04-2005



Printed: 22-04-2005 TIPA1 99910592

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

458 20-04-2005

GENE SILENCING

This invention relates to the control of gene expression, more particularly to the inhibition of expression, commonly referred to as "gene silencing".

Two principal methods for the modulation of gene expression are known. These are referred to in the art as "antisense downregulation" and "sense downregulation"(also, referred to as "cosuppression"). Both of these methods lead to an inhibition of expression of the target gene.

In antisense downregulation, a DNA which is complementary to all or part of an endogenous target gene is inserted into the genome in reverse orientation. While the mechanism has not been fully elucidated, one theory is that transcription of such an antisense gene produces mRNA which is complementary in sequence to the mRNA product transcribed from the endogenous gene: that antisense mRNA then binds with the naturally produced "sense" mRNA to form a duplex which inhibits translation of the natural mRNA to protein. It is not necessary that the inserted antisense gene be equal in length to the endogenous gene sequence: a fragment is sufficient. The size of the fragment does not appear to be particularly important. Fragments as small as 42 or so nucleotides have been reported to be effective. Generally somewhere in the region of 50 nucleotides is accepted as sufficient to obtain the inhibitory effect. However, it has to be said that fewer nucleotides may very well work: a greater number, up to the equivalent of full length, will certainly work. It is usual simply to use a fragment length for which there is a convenient restriction enzyme cleavage site somewhere downstream of fifty nucleotides. The fact that only a fragment of the gene is required means that not all of the gene need be sequenced. It also means that commonly a cDNA will suffice, obviating the need to isolate the full genomic sequence.

The antisense fragment does not have to be precisely the same as the endogenous complementary strand of the target gene. There simply has to be sufficient sequence similarity to achieve inhibition of the target gene. This is an important feature of antisense technology as it permits the use of a sequence which has been derived from one plant species to be effective in another and obviates the need to construct antisense vectors for each

Printed: 22-04-2005 TIPAT 99910592

individual species of interest. Although sequences isolated from one species may be effective in another, it is not infrequent to find exceptions where the degree of sequence similarity between one species and the other is insufficient for the effect to be obtained. In such cases, it may be necessary to isolate the species-specific homologue.

Antisense downregulation technology is well-established in the art. It is the subject of several textbooks and many hundreds of journal publications. The principal patent reference is European Patent No. 240,208 in the name of Calgene Inc. There is no reason to doubt the operability of antisense technology. It is well-established, used routinely in laboratories around the world and products in which it is used are on the market.

Both overexpression and downregulation are achieved by "sense" technology. If a full length copy of the target gene is inserted into the genome then a range of phenotypes is obtained, some overexpressing the target gene, some underexpressing. A population of plants produced by this method may then be screened and individual phenotypes isolated. A similarity with antisense is that the inserted sequence need not be a full length copy. The principal patent reference on cosuppression is European Patent 465,572 in the name of DNA Plant Technology Inc. There is no reason to doubt the operability of sense/cosuppression technology. It is well- established, used routinely in laboratories around the world and products in which it is used are on the market.

Sense and antisense gene regulation is reviewed by Bird and Ray in Biotechnology and Genetic Engineering Reviews 9: 207-227 (1991). The use of these techniques to control selected genes in tomato has been described by Gray et.al., Plant Molecular Biology, 19: 69-87 (1992).

Gene silencing can therefore be achieved by inserting into the genome of a target organism an extra copy of the target gene coding sequence which may comprise either the whole or part or be a truncated sequence and may be in sense or antisense orientation. Additionally, intron sequences which are obtainable from the genomic gene sequence may be used in the construction of suppression vectors. There have also been reports of gene silencing being achieved within organisms of both the transgene and the endogenous gene where the only sequence identity is within the promoter regions.

455 20-04-2005

Printed: 22-04-2005 TIPA 99910592

Gene control by any of the methods described requires insertion of the sense or antisense sequence, under control of appropriate promoters and termination sequences containing polyadenylation signals, into the genome of the target plant species by transformation, followed by regeneration of the transformants into whole plants. It is probably fair to say that transformation methods exist for most plant species or can be obtained by adaptation of available methods.

The most widely used method is *Agrobacterium*- mediated transformation, mainly for dicotyledonous species. This is the best known, most widely studied and, therefore, best understood of all transformation methods. The rhizobacterium *Agrobacterium tumefaciens*, or the related *Agrobacterium rhizogenes*, contain certain plasmids which, in nature, cause the formation of disease symptoms, crown gall or hairy root tumours, in plants which are infected by the bacterium. Part of the mechanism employed by *Agrobacterium* in pathogenesis is that a section of plasmid DNA which is bounded by right and left border regions is transferred stably into the genome of the infected plant. Therefore, if foreign DNA is inserted into the so-called "transfer" region (T-region) in substitution for the genes normally present therein, that foreign gene will be transferred into the plant genome. There are many hundreds of references in the journal literature, in textbooks and in patents and the methodology is well-established.

Various methods for the direct insertion of DNA into the nucleus of monocot cells are known.

In the ballistic method, microparticles of dense material, usually gold or tungsten, are fired at high velocity at the target cells where they penetrate the cells, opening an aperture in the cell wall through which DNA may enter. The DNA may be coated on to the microparticles or may be added to the culture medium.

In microinjection, the DNA is inserted by injection into individual cells via an ultrafine hollow needle.

Another method, applicable to both monocots and dicots, involves creating a suspension of the target cells in a liquid, adding microscopic needle-like material, such as silicon carbide or silicon nitride "whiskers", and agitating so that the cells and whiskers collide and DNA present in the liquid enters the cell.

456 20-04-2005

In summary, then, the requirements for gene silencing using both sense and antisense technology are known and the methods by which the required sequences may be introduced are known.

The present invention aims to, *inter alia*, provide a method of enhancing the control of gene expression.

According to the present invention there is provided a vector for enhancing the inhibition of a selected target gene within an organism, comprising a gene silencing vector characterised in that the said gene silencing vector includes a inverted repeat of all or part of a polynucleotide region within the vector.

The inverted repeat sequence may be a synthetic polynucleotide sequence and its inverted repeat sequence or an inverted repeat of all or part of the said gene silencing vector or an inverted repeat of the 5'-untranslated region of the gene silencing vector.

The inverted repeat may be separated from the polynucleotide region by a sequence of nucleotides.

The invention also provides a method of controlling the expression of a DNA sequence in a target organism, comprising inserting into the genome of said organism an enhanced gene silencing vector as defined above.

In a preferred embodiment a vector for enhanced gene silencing comprising in sequence a promoter region, a 5'-untranslated region, a transcribable DNA sequence and a 3'-untranslated region containing a polyadenylation signal, characterised in that the said construct includes an inverted repeat of a region of said vector.

It is preferred that the inverted repeat is a fragment of the 5'-untranslated region of the said vector. The vector may have two tandem copies of the inverted repeat.

In simple terms, we have found that the inhibitory effect of a gene-silencing vector can be enhanced by creating in the vector an inverted repeat of a part of the sequence of the vector. Alternatively the inverted repeat may be of a synthetic sequence which may be created independently of the vector itself and then inserted into the vector sequence. While the mechanism by which the enhancement is achieved is not fully understood we understand that the minimum required for such a vector is a region or regions which identify the gene targeted for silencing and an inverted repeat of a part of that region or, as explained above an inserted sequence and its inverted repeat. The region of the vector which identifies the gene

targeted for silencing may be any part of that endogenous gene which characterises it, for example, its promoter, its 5'-untranslated region, its coding sequence or its 3'untranslated region. We have also found that the vector used in this invention will silence the expression of the target gene and also any members of the gene family to which the targeted gene belongs.

Although the mechanism by which the invention operates is not fully understood, we believe that creation of an inverted repeat promotes the formation of a duplex DNA between the selected sequence and its inverted.

The inverted repeat may be positioned anywhere within the vector such as within the promoter region, the 5' untranslated region, the coding sequence or the 3' untranslated region. If the inverted repeat is based on a contiguous sequence within the promoter region, then it is preferred that the inverted repeat in located within the promoter region. If the inverted repeat is based on a contiguous sequence within the 5' untranslated region, then it is preferred that the inverted repeat is located within the 5' untranslated region. If the inverted repeat is based on a contiguous sequence within the coding region, then it is preferred that the inverted repeat is located within the coding region. If the inverted repeat is based on a contiguous sequence within the 3' untranslated region, then it is preferred that the inverted repeat is located within the 3' untranslated region.

The selected polynucleotide sequence and its inverted repeat may or may not be separated by a polynucleotide sequence which remains unpaired when the 5' untranslated region and the inverted repeat have formed a DNA duplex. It is preferred however, that the chosen contiguous sequence and its inverted repeat are separated by a polynucleotide sequence which remains unpaired when the 5' untranslated region and the inverted repeat have formed a DNA duplex.

It is further preferred that the inverted repeat is based on the 5' untranslated sequence. It is also preferred that the inverted repeat is positioned upstream of the coding sequence. It is further preferred that the inverted repeat is positioned between the 5' untranslated region and the coding sequence. It is further preferred that the 5' untranslated region and the inverted repeat are separated by a polynucleotide sequence which remains unpaired when the 5' untranslated region and the inverted repeat have formed a DNA duplex.

Suppression can also be achieved by creating a vector containing an inverted repeat sequence which is capable of forming a duplex DNA within the promoter region of the target gene. This obviates the need to include any specific coding sequence information about the gene to be suppressed since the vector would allow suppression of the promoter within the organism and hence the expression of the target gene. Alternatively vectors may be created which are lacking a promoter sequence but which contain an inverted repeat of a sequence within the 5' untranslated region, the coding region or the 3' untranslated region.

The 5' or 3' untranslated regions of a gene suppression vector can also be replaced with a synthetic 5' or 3' untranslated regions which comprises a polynucleotide part and inverted repeat separated by a polynucleotide sequence which remains unpaired when the said polynucleotide part and the inverted repeat form a DNA duplex. It is preferred to construct a synthetic 5' untranslated region. It is further preferred to construct the synthetic 5' untranslated region comprising sequentially, a 33 base polynucleotide part and a 33 base polynucleotide inverted repeat separated by a 12 base polynucleotide.

Where it is desired to use an inverted repeat sequence within the 5' untranslated region, the coding sequence or the 3' untranslated region, gene silencing vectors constructed with inverted repeats within any one of these regions may additionally enable the silencing of genes that are homologous to the coding sequence present in the silencing vector. Therefore when it is desired to silence genes homologues within an organism the construction of a silencing vector containing an inverted repeat within the 5' untranslated region, the coding sequence or the 3' untranslated region may allow the silencing of all the genes exhibiting sequence homology to the coding sequence within the construct. Homology/homologous usually denotes those sequences which are of some common ancestral structure and exhibit a high degree of sequence similarity of the active regions. Examples of homologous genes include the ACC-oxidase enzyme gene family which includes ACO1 and ACO2.

Any of the sequences of the present invention may be produced and manipulated using standard molecular biology techniques. The sequences may be obtained from a desired organism source such as plant sources and modified as required or synthesised *ab initio* using standard oligosynthetic techniques.

Printed: 22-04-2005 TIPA1 99910592

Without wishing to be bound by any particular theory of how it may work, the following is a discussion of our invention. 96% of tomato plants transformed with an ACC-oxidase sense gene containing two additional, upstream inverted copies of its 5' untranslated region, exhibited substantially reduced ACC-oxidase activity compared to wild type plants. Only 15% of plants transformed with a similar construct, without the inverted repeat, had reduced ACC-oxidase activity. Both populations had similar average numbers of transgenes per plant. Treatment of tomato leaves with cycloheximide caused a strong, reproducible increase in the abundance of ACC-oxidase transcripts and was used in the study of suppression by ACC-oxidase sense transgenes in preference to wound induction used in previous studies. The relative abundance of unprocessed and processed ACC-oxidase transcripts in suppressed and non-suppressed plants was assayed by ribonuclease protection assays, providing an indirect measure of transcription and mRNA accumulation which did not rely upon assaying isolated nuclei. This analysis indicated that the suppression of ACO1 gene expression was mainly post-transcriptional. Using the same type of RPA assay similar results were obtained from plants containing suppressing polygalactronase-sense or ACO-antisense transgenes.

There are now numerous examples of the inactivation of homologous sequences in plants. The term "homology dependent gene silencing" (HDGS) best describes all of these although it should be noted that in most examples the "silencing" is not complete and a low level of gene expression remains. Throughout this specification we will use the classification most-recently outlined by Matzke and Matzke, Plant Physiol. 107: 679-685 (1995) in which different examples of HDGS were divided into three main groups; cis-inactivation, trans-inactivation, and sense-suppression. Down regulation by antisense genes bears many similarities to the last of these and has been suggested to operated by the same mechanism (Grierson et al, Trends Biotechnol. 9: 122-123 (1991)). Both sense and antisense transgenes have been widely used to reduce the expression of homologous endogenous genes in plants. Although the underlying mechanisms of HDGS remain obscure, this technology has found numerous applications not only in fundamental research but also in commercial biotechnology ventures and new food products are already on the market.

At present , obtaining a large number of strongly suppressed, transgenic lines is more a matter of luck than judgement. A positive correlation between the presence of repeated

460 20-04-2005

transgene sequences and the incidence of HDGS has been noted. However single locus-transgene insertions associated with HDGS have also been reported.

There is an emerging consensus that different examples of HDGS can be classified on the basis of whether or not the transcription of the target gene is affected. Examples of transcriptional suppression have been described. Where the homology between interacting genes resides within transcribed sequences, HDGS has been shown to be a post-transcriptional effect. Despite this apparently precise demarcation, several similarities exist between some examples in the two different categories. These include variegated patterns of silencing, increased methylation of genes participating in silencing and the frequent observation that silencing loci contain repeated sequences.

Although transcriptional silencing must occur in the nucleus, post-transcriptional silencing might occur in either or both the nucleus or cytoplasm. There is evidence that the abundance of processed, nuclear RNA of silenced genes was unaffected and suggested an effect upon transport into or degradation within the cytoplasm. More compelling evidence that post-transcriptional HDGS occurs outside the nucleus is the relationship between gene silencing involving nuclear transgenes and resistance to cytoplasmically replicating RNA viruses. Transgenic plants containing transgenes that suppress the activity of other transgenes (e.g. GUS) or endogenous genes (e.g. PG) are also resistant to RNA viruses which have been engineered to include sequences from those genes. Nevertheless, nuclear features such as transgene methylation and complexity of transgene loci were found positively to correlate with virus resistance. In almost all instances of HDGS, the source of the silencing is nuclear (even if the manifestation is cytoplasmic). However, silencing of a nuclear gene by a cytoplasmic element has been demonstrated by the suppression of phytoene desaturase in plants infected by a recombinant virus containing sequences from that gene.

Although, there are now numerous examples of post-transcriptional suppression of plant genes by HDGS, as yet, there is no information as to whether the increased turnover of pre-mRNA is related to or distinct from other cellular, RNA turnover processes. Degradation of RNA in plants is poorly understood but there is evidence that translation is involved. For example, the very short half lives (around 10 minutes) of small auxin up RNAs (SAURS) can be markedly prolonged by treatment with cycloheximide.

Printed: 22-04-2005 TIPAT 99910592

This invention gives a striking increase in the frequency of HDGS following the inclusion of a short repeated region within a transgene. Expression of the target gene encoding the terminal ethylene biosynthetic enzyme ACC-oxidase, in tomato was suppressed by such constructs mainly post-transcriptionally. This was shown to be true for other examples of sense and antisense suppression in tomato. Cycloheximide was found to be a potent and reliable inducer of ACO gene expression but did not ameliorate the silencing.

The invention will now be described, by way of illustration, in the following Examples and with reference to the accompanying Figures of which:

Figure 1. (A) ACO1 gene silencing vector.

(B) ACO1 gene silencing vector containing tandem inverted repeats of the 5' untranslated region.

Figure 2. Illustrates the relative ACC-oxidase activity in both types of transgenic plant relative to wild type values where C = transgenic plants containing construct C (Figure 1A) and V = transgenic plants containing construct V (Figure 1B).

Figure 3. Tomato plant ACC Oxidase activity of transgenic transformants containing pHIR-ACO (as illustrated in SEQ ID No 10). The graph also includes C12ACO (overexpression control) an untrasformed wild type and TOM13 strong antisense gene silenced control.

Example 1.0

Construct V (Figure 1) was made in the following manner: 79 base pairs of the 5' untranslated region of the tomato ACO1 cDNA was amplified by PCR and two copies were ligated in tandem in the reverse orientation immediately upstream of the ACO1 cDNA which contains its own polyadenylation signal in its 3' untranslated region (construct C). Both were ligated downstream of the CaMV 35S promoter and then transferred to the binary vector, Bin19. Figure 1 shows the basic details of constructs "C" and "V". These were used to transform tomato plants (Ailsa Craig) by *Agrobacterium* mediated DNA transfer. 13 and

462 20-04-2005

28 individual kanamycin resistant calli were obtained with constructs "C" and "V" respectively and these were regenerated into plants.

The nucleotide sequence of the promoter and 5' untranslated region of the ACO1 gene is given as SEQ ID NO 1 hereinafter. The 79bp referred to above begins at base number 1874 and stops at the base immediately preceding the translation start codon (ATG) at number 1952.

Example 1.1

To screen the population for any effects on ACO gene expression, relative ACO activity was measured from untransformed and transformed plants. The production of ethylene from leaf discs supplemented with the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, was measured at least three times from each plant. The cutting of the discs by a cork borer wounds the leaves and stimulates the expression of the ACO1 gene. ACC-oxidase activity in both types of transgenic plant relative to wild type values are shown in Figure 2. There was a dramatic difference in ACO activity between the two populations, with plants containing the inverted repeat (V line) showing very strong suppression. The majority (11 out of 13) of plants of the C line did not show suppression of ACO activity but overexpression, compared to wild-type plants, as would be expected since this construct contained a translatable ACO1 coding sequence.

To test for the presence of the transgenic ACO sequence, DNA from the plants was analysed by PCR using two oligos homologous to and complementary with the beginning and end respectively of the ACO1 coding sequence. This combination co-amplifies 1500 bp of the endogenous ACO1 gene (which acts as an internal positive control) and the ACO1 sense transgene as a 1000 bp fragment (since it was derived from a cDNA and so has no introns). The amplified region does not include the repeated region of the V-type transgene. The two fragments were separated by gel electrophoresis and detected by staining with ethidium bromide. This showed the presence of the transgene in all plants of the C line and all plants of the V line except one (V2) which also had no reduced ACC-oxidase activity (Figure 2).

Printed: 22-04-2005 TIPAT 99910592

Example 1.2

It was considered possible that the repeated region in the transgene might have affected the number of transgenes which integrated into the genome and that this was the actual source of high frequency silencing. The PCR assay described above can be used to estimate the transgene copy number if the following assumptions are made:

1) that in any transgenic plants there was no variation in the number of endogenous ACO1 genes per genome;

2) that the amplification efficiency ratio (endogenous ACO1 DNA: transgenic ACO1 DNA) is constant;

3) the reaction is sampled at low DNA concentration to minimise product re-annealing. Since we were only concerned with estimating the number of transgenes in the two lines relative to each other and not absolute quantification of transgene copy number, we did not employ synthetic combinations of "transgene" and "endogenous gene" DNA as standards.

After 20 cycles of amplification, gel-electrophoresis, Southern blotting, and hybridisation with a radioactively labelled ACO1 cDNA, the signal from endogenous and transgenic ACO1 DNA was visualised and quantified by phoshorimaging. The average transgene: endogenous gene ratio for the C line was 0.96 and for the V line 1.08 indicating that the repeat region in the V construct does not cause more T-DNAs to integrate during transformation.

Example 1.3

ACO1 mRNA increased in abundance following wounding and/or treatment of leaves with cycloheximide but accumulation was approximately five times greater after treatment with cycloheximide than after mechanical wounding which we have previously used as a stimulus. Wounding of cycloheximide treated leaves failed to elicit a further increase in ACO1 mRNA amount. We found cycloheximide to be a more reproducible inducer of ACO1 mRNA accumulation than mechanical wounding and so have used it in preference to

464 20-04-2005

Printed: 22-04-2005 TIPAT 99910592

the latter in this study. No further increase in the abundance of ACO1 mRNA was observed when the concentration of cycloheximide was increased from 50 to 250 ug/ml (date not shown).

Example 1.4

The 5' end of ACO1 mRNA extracted from plants is heterogeneous but consists of two major species which differ by 2 bases. The 5' untranslated region (both the sense and duplicated antisense sequences) in both of the constructs (C and V) was made approximately 10 base pairs shorter than those of the endogenous gene. This allowed the discrimination of endogenous gene and transgene-derived transcripts by ribonuclease protection assays using a probe transcribed from a genomic ACO1 sequence which extended from the start of the 3' end of the 5' untranslated region to a AccI site, in the promoter of ACO1, 222 bases upstream. In RNA from wild type leaves, there were several bands which may arise from distinct RNA species or from breaking of RNA duplexes during digestion. Some of the bands seem more susceptible to the effects of antisense suppression than others (although the general trend is still suppression).

In leaves from lines V4, V11 and V28 (all <10% ACO activity), there was extensive co-suppression of the endogenous transcripts (relative to wild-type) and the transgene transcripts (relative to those from a control transgene (line C1). V4, V11 and V28 all exhibited greater suppression than the homozygous ACO-antisense line (Hamilton et.al. Nature 346, 284-287(1990)).

The use of the protein synthesis inhibitor cycloheximide as a stimulant of ACO1 RNA accumulation did not obviously alleviate the suppression of this RNA by the sense transgenes in lines V4, V11 or V28.

Although the endogenous genes transcript is unquestionably suppressed, it is possible that the inverted repeat within the 5' end of the V transgene transcript excludes the probe and causes the signal from the transgenic RNA to be underrepresented. This seems unlikely for the following reason. When a probe that was not excluded by the inverted repeat was used to analyse RNA from the V line, the mRNA signal (which, using this probe, is actually the sum of the endogenous and the transgenic RNAs) was still much less than in the wild type. The

65 20-04-2005

Printed: 22-04-2005

TIPAT

99910592

data shows that in the absence of silencing, the abundance of the endogenous and transgenic RNAs are comparable.

Example 1.5

We chose to measure the abundance of unprocessed transcripts in total RNA extracts as a indirect measurement of transcription whilst simultaneously measuring the amount of processed mRNA. This was achieved using RNA probes transcribed from genomic sequences spanning introns in ribonuclease protection assays. Since the RNA analysed was from leaves frozen in liquid nitrogen and then extracted in strongly protein-denaturing conditions (phenol and detergent) there should have been little opportunity for any resetting of transcription during the process There was a greater abundance of mRNA following treatment with cycloheximide although the total amount of mRNA in the ACO-AS plants was reduced. In the ACO-sense line, V11, there was little or no increase in the mRNA signal. It is likely that this mRNA signal is mainly from the transgene which is transcribed by the 35S promoter which is not cycloheximide inducible. In contrast, the abundance of the primary transcript in all RNA samples increased following cycloheximide treatment. This RNA species originates only from the endogenous ACO1 gene since the transgene has no introns. In all cases the suppressing transgene had little or no effect upon the abundance of the primary transcript.

Example 1.6

Cycloheximide strongly stimulated the accumulation of both the ACO1 primary transcript and mature mRNA. Quantification of the signal from primary transcripts and mature ACO1 RNA in wild type leaves before and after treatment with cycloheximide showed that there was a 6 fold increase in the abundance of unprocessed ACO1 RNA but a 13 fold increase in the amount of processed ACO1 RNA. The abundance of transgenic ACO1 RNA (transcribed from the 35S promoter) in the C line also rose upon treatment with cycloheximide.

Example 1.7

166

20-04-2005

Two tandemly linked copies of the 5'UTR (each unit = 79bp; 74.7% (A+T)) were litigated in the inverted orientation between the CaMV 35S promoter and an almost full length ACO1 cDNA (Figure 1). Either unit of this direct repeat has the capacity to form a large cruciform structure with the 5'untranslated region immediately downstream. After *Agrobacterium*-mediated transformation with this construct, 26 out of 28 plants recovered from tissue culture exhibited suppressed ACO activity. A much lower frequency (2/15) of suppression was observed with a control construct which lacked the duplicated 5'UTR but was otherwise the same.

More transgenic plants were obtained with the V construct than with the control construct (as well as exhibiting the high HDGS frequency). It is likely that this is a direct result of reduced ethylene synthesis as a result of ACO gene suppression. Previous results have shown that greatly improved callus regeneration could be achieved after transformation with constructs which contained an ACO-antisense gene.

Of the two plants transformed with the repeat construct that showed no suppression, one, V2, may have had a truncated T-DNA or be an untransformed escape since the transgenic ACO1 sequence could not be amplified. Since the repeat contained DNA sequences already in the gene, it seems unlikely that it is this sequence per se which elicits the effect upon gene silencing. It is much more likely that it is the structure of the repeat DNA (or the transcribed RNA) which is the source of the high frequency of silencing observed. The repeat within the V construct was similar to that with the control construct

Most instances of HDGS are associated with complex transgenic loci that contain repeats or whole or part T-DNAs rather than simple single insertions but it is not known whether this is a primary determinant of suppression or an indirect effect. There are examples where apparently single transgenes are associated with gene silencing but these are in the minority and in at least some of these examples the T-DNAs contain internal repeats. The data presented here suggest that deliberate introduction of small repeats in a transgene can increase the number of transgenic lines in which homologous genes have been suppressed to almost 100%. Sense suppression could be obtained with the control construct but at a much lower frequency. The deliberate introduction of repetitive DNA into a transgene may substitute for a requirement for the insertion of repeated T-DNA units to

Printed: 22-04-2005 TIRA1 99910592

produce silencing. Although the PCR assay used here is not absolutely quantitative, it does suggest that the average transgene dosage is about 2 implying that some of the lines exhibiting suppression have single insertions. In several of our lines, the suppression obtained is profound (Figure 2) which makes this strategy even more attractive to those interested in specifically switching off gene expression. There is one previous report of the deliberate combination of repetitive DNA with a reporter gene effecting increased HDGS: Lohuis et al., Plant Journal, 8, 919-932 (1995) inserted a copy of a randomly isolated repetitive genomic sequence (RPS) upstream of GUS reporter gene and found that this element increased the frequency of variegation of transgene expression. This is an example of cis-inactivation, probably acts at the transcriptional level, and the authors considered it to be distinct from co-suppression/sense-suppression phenomena. Interestingly, the RPS element did not increase the frequency of complete silencing of the transgene. In our example, although the level of suppression is severe in many lines, it is not possible to say whether the degree of suppression is equal in all cells expressing the target gene or if the repeat has simply greatly increased the proportion of cells experiencing suppression.

Example 1.8

Constructs and transformation

The tomato ACO1 cDNA, pTOM13 was released from its original cloning vector, pAT153, (Promega), creating pG31. pG31 was digested with EcoRI and the vector re-ligated to create pTRD. This removed the 5' end of the cDNA which contains approximately 90 base pairs of the 3'untranslated region in the antisense orientation at its 5' end which may have been introduced artefactually during the original cloning of the pTOM13 cDNA . The remaining ACO1 sequence was cut out from pTRD with EcoRI and HindIII and ligated into $pT_7.T_{3\alpha}18$ (BRL) digested by EcoRI and the ends filled in with Klenow enzyme. The 5' untranslated region of the ACO1 transcript (minus approximately 10 bases at the 5' end) was amplified with Taq polymerase from oligo dT-primed cDNA of wounded tomato leaves with the primers 5' CATTCATCTCTTCAATCTTTTG 3' (SEQ ID No.2) and 5' CTTAATTTCTTGGTAAAGTGTTTTCC 3' (SEQ ID NO.3). This DNA was rendered flush ended with T4 DNA polymerase and ligated with the filled in pTRF to create pMI1. This reconstituted the EcoRI site at the 5' end and yielded a translatable ACO1 cDNA

Ads 20-04-2005

slightly shorter than the wild type ACO1 mRNA. Sequencing confirmed that the amplified ACO1 sequence was not mutated. pMI1 was digested with HindIII and partially with EcoRI and the fragment containing the ACO1 cDNA sequence was filled in with Klenow enzyme, and ligated with SmaI digested pDH51 to create pDHC1. This was digested with XbaI and HindIII, the filled in and the fragment containing the vector, 35S promoter and ACO1 cDNA religated to create pMI5. pMI7 contains two copies of the 5'UTR of ACO1 tandemly linked and inserted in the antisense orientation upstream of the 5'UTR of ACO1 in pMI5. This was made by amplifying the 5'UTR from tomato leaf cDNA (see above) with oligos 5' CATTCATCTCTTCAATCTTTTG 3' (SEQ ID No.2) and 5'CTTAATTTCTTGGTAAAGTGTTTTCC 3'(SEQ ID NO.3)., polishing the DNA with T4 DNA pol and ligating it into a filled in Acc651 site in pMI5 upstream of the 5'UTR of the ACO1 sequence Acc651 (an isoshizomer of KpnI but which gives a 5' overhang). The construction was confirmed by sequencing.

pDHC1 and pMI7 were digested with BamHI, BglI and PvuII and the BamHI-PvuII fragments containing the CamV35S-ACO1cDNA sequences were cloned into Bin19 which had been cut by HindIII, filled in and then cut by BamHI. The resulting recombinants were called pBC1 and pBM17 respectively. These plasmids were transformed into *A. tumefaciens* LBA4404: and this used to transform tomato cotyledons (*Lycopersicon esculentum* var Ailsa Craig). Plants were regenerated from callus grown on 50$\mu g.ml^{-1}$ kanamycin.

Example 1.9

ACC-oxidase assays

ACC-oxidase activity was measured as the ability of plant tissue to convert exogenous 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene. Discs were cut from leaf lamina with a sharp cork borer and placed in contact with 0.5ml of 10mM NaH_2PO_4/Na_2HPO_4 (pH7), and 10 mM ACC (Sigma) in 5 ml glass bottles which were then sealed with "Subaseal" vaccine caps (Fisons). After 1 hour at room temperature, the ethylene in the head space was measured by gas chromatography as described by Smith et al., 1986. Ethylene was also measured from bottles containing the solution but without leaf

tissue. These values were subtracted from the values obtained from the bottles containing leaf discs.

Example 1.10

PCR analysis of transgenic plants

DNA was extracted from singles leaves of wild type plants, plants homozygous for a ACO-antisense gene, and those transformed with the constructs of pBC1 and pBM17. Leaves were frozen in liquid nitrogen, briefly ground in eppendorf tubes with a disposable pipette tip, ground further after the addition of 200µl DNA extraction buffer (1% laurylsarcosine, 0.8% CTAB, 0.8M NaCl, 0.02M EDTA, 0.2M Tris/HCl (pH8)), heated to 65°C for 15 minutes, extracted once with phenol/chloroform and the DNA precipitated from the aqueous phase by the addition of 0.6 volumes of isopropanol. The DNA was recovered by centrifugation, the pellets washed in 70% ethanol, dried and redissolved in 200ul, of TE buffer. 1ul of this was used as template for simultaneous PCR amplification of the endogenous ACO1 gene and the transgene using the primers ACO1.1 (ATGGAGAACTTCCCAATTATTAACTTGGAAAAG SEQ ID NO 4) and the ACO1.2 (CTAAGCACTTGCAATTGGATCACTTTCCAT SEQ ID NO 5) for 21 cycles of 30 seconds at 95°C, 30 seconds at 65°C and 1 minute at 72°C. Amplified DNA was separated by electrophoresis in a 0.8% agarose/1xTBE gel and blotted onto HybondN+ in 0.4M NaOH for 6 hours. To detect the amplified ACO sequences, the DNA on the filter was hybridised with random prime labelled ACO1 cDNA. The filter was washed in 0.2xSSPE/1%SDS at 65°C followed by phosphorimaging of the radioactive signal.

Example 1.11

Treatment of leaves with cycloheximide and mechanical wounding

Compound leaves were excised with a sharp scalpel blade and immediately placed under water solution of 50µl.ml^{-1} cycloheximide (Sigma). Another 3 cm of the stalk was cut from the branch under the solution and the assembly was then left in a laminar airflow for six hours to allow the cycloheximide to enter the leaves.

Printed: 22-04-2005 TIPA1 99910592

To wound leaf tissue, individual leaflets were placed on a hard surface and diced with a sharp scalpel blade approximately 10 times transversely and 5 times longitudinally.

Example 1.12

Northern analysis of ACO mRNA in leaves treated with cycloheximide

RNA was extracted from cycloheximide treated leaves as follows. Tissue was frozen in liquid nitrogen and pulverised either in a coffee grinder (for fruit pericarp, see below) or in a mortar (for leaves). 5ml.gfwt^{-1} of RNA extraction buffer (Kirby's) was added and the frozen slurry ground further in disposable polypropylene centrifuge tubes with a glass rod. Once thawed, the mixture was extracted twice with phenol/chloroform and the nucleic acids precipitated by the addition of 2.5 volumes of ethanol, 1/10 volume 3M sodium acetate (pH5) and refrigeration at 20°C for 1 hour. After centrifugation at 3000xg for 10 minutes (40 minutes for a fruit extraction), the pellets were redissolved quickly in water (approximately 1ml per gram of tissue) and, an equal vol. of 2x DNA extraction buffer (1.4M NaCl, 2% CTAB, 100mM Tris/HCl (pH8)). Two volumes of precipitation buffer (1%CTAB, 50mM Tris/HCl (pH8)) were added to precipitate the nucleic acids (30 minutes at room temparature suffices) and the precipitate was collected by centrifugation (3000xg/15 minutes). This step was repeated except the pellets were dissolved in 1xDNA extraction buffer. After collection of the second precipitation, the pellets were redissolved in 0.5ml 1M NaCl and immediately reprecipitated with 2.5 volumes of ethanol (-20°C/30 minutes). After centrifugation (10000xg/10 minutes), the pellets were redissolved in 400µl water and extracted twice with phenol/chloroform. The nucleic acids were precipitated and collected as above redissolved in 400µl water. 46ul of 10 x One-Phor-All-Buffer (Pharmacia) was added with 50 units of RNAase-free DNAase (Promega) and the solutions incubated at 37°C for 30 minutes. They were extracted twice with phenol/chloroform, the RNA precipitated and collected as above and finally redissolved in 100-500ul of water. We have found that this relatively extensive purification is necessary if rare transcripts are to be detected by RPA. Also, the RNA re-dissolves readily which greatly reduces handling time when manipulating this RNA mixed with radioactive probe RNA.

47:1 20-04-2005

Printed: 22-04-2005 TIPA 99910592

50μg of leaf RNA was mixed with an equal volume of denaturation/loading solution (50% formamide; 25mM sodium phosphate (pH6.5); 10mM EDTA; 6.2% formaldehyde; 200μg.ml^{-1} ethidium bromide) and separated by electrophoresis on a 25mM sodium phosphate (pH6.5) /3.7% formaldehyde /1.5% agarose gel in 10mM sodium phosphate (pH6.5)/3.7% formaldehyde with continuous buffer re-circulation. The separated RNA was blotted onto Genescreen (Dupont) hybridisation membrane in 10mM sodium phosphate (pH6.5). The autocrosslink setting on a Stratalinker (Stratagene) was used to covalently link the RNA to the filter. The filter was prehybridised and then hybridised with a 32P-random prime labelled ACO1 cDNA probe. The filter was washed in 0.2xSSPE/1%SDS at 65°C and then exposed to Kodak X-omat film between two intensifying screens at -70 for 24 hours. Subsequently the radioactivity in each band was measured by phophorimaging.

Example 1.13

Ribonuclease protection analysis

RNA was extracted from cycloheximide treated leaves and fruit described above.

RNA probes were transcribed with T7 RNA polymerase at 20°C with α-^{32}P UTP (400Ci. mmol^{-1}) as the sole source of UTP. After 1 hour incubation, RNAase-free DNAase was used to remove the template and the probe was further purified on 6%polyacrylamide/8M urea/1xTBE gels. The band containing the full length probe was visualised by autoradiography. The gel slice containing this RNA was excised and placed in 1ml probe elution buffer (0.5M ammonium acetate; 1mMEDTA; 0.2% SDS) for between 6 and 14 hours at 37°C. Typically, between 20m and 100μl of this would be co-precipitated with between 20 or 100μg of the RNA to be tested plus two yeast RNA controls. The precipitated RNAs were redissolved in 30μl hybridisation solution (80% formamide; 40mM PIPES/NaOH; 0.4M sodium acetate; 1mM EDTA pH should be 6.4) heated to 65°C for 10 minutes and hybridised at 42°C for between 2 to 14 hours. The longer hybridisation times were purely for convenience since we easily detected even rare transcripts after only 2 hours of hybridisation. 300μl of RNAase digestion buffer (5mM EDTA; 200mM sodium acetate; 10mM Tris/HCl. Final pH of solution should be 7.5) containing either RNAaseONE (Promega) or RNAase T1 (Ambion) was added to each tube except one containing yeast RNA which received RNAase digestion buffer without any ribonuclease. Incubation of the

20-04-2005

digesting RNA was at either 25°C (RNAaseONE) or 37°C RNAaseT1) for 2-4 hours. RNAaseONE was inactivated by the addition of SDS to 0.5% and the protected, double stranded RNAs were precipitated with ethanol and sodium acetate. RNAaseT1 was inactivated and the double stranded RNAs were precipitated by the addition of the inactivation/precipitation solution provided with the RNAase protection kit from Ambion. The protected RNAs were redissolved in 5-10ul of denaturation/loading solution (80% formamide; 10mM EDTA; 0.1% bromophenol blue; 0.1% xylene cyanol; 0.1% SDS), heated to 95°C for 5 minutes and then separated by electrophoresis on a on 6-8% polyacrylamide/8M urea/1xTBE gels (the concentration of polyacrylamide depending on upon the sizes of the fragments to be separated). After electrophoresis, the gels were dried and exposed to Kodak x-omat film between two intensifying screens at -70 for the time indicated. The radioactivity was measured by phosphorimaging.

EXAMPLE 2.0

Construction of synthetic heterologous DNA inverted repeat.

A synthetic heterologous DNA invert repeat (SEQ ID No 11) was constructed by annealing two sets of synthetic oligos (HIR1 SEQ ID No 12 and HIR2 SEQ ID No 13 and HIR 3 SEQ ID No 14 and HIR 4 SEQ ID No 15) and ligating each set into pSK-(bluescript, Statagene) independently, to create pHIRA and pHIRB respectively. The invert repeat structure was created by digesting both pHIRA/B vectors with XhoI and NcoI and ligating the 42bp fragment from pHIRB into the pHIRA. The invert repeat structure was isolated from the pSK- vector using KpnI and cloned into the KpnI site immediately downstream of the CaMV35S promoter in the plant expression cassette pSIN to create pHIR-SIN.

The tomato ACO1 cDNA (pTOM13) coding sequence was amplified from its original cloning vector pAT153 (promega) using two oligonucleotide primers, 5' CTTTACCAAGAAGTGCACATGGAGAACTTCCC 3'SEQ ID No 6, and 5'GAATTGGGCCCTAAGCACTTGCAATTGG 3' SEQ ID No 7 which prime either side of the TOM13 coding sequence introducing ApaLI and ApaI sites respectively. The PCR product was digested with ApaLI and ApaI and the ends blunted in using Pfu polymerase

Printed: 22-04-2005 TIPA 99910592

(Stratagene). The blunt PCR fragment was ligated into the SmaI site downstream of the invert repeat structure of pHIR-SIN to create pSIN-HIR-ACO.

The plant expression cassette from pHIR-ACO was isolated using AgeI and ligated into the binary vector pVB6 AgeI site to create pHIR-ACO SEQ ID No 10. The insert was orientated using restriction analysis to ensure that all the ORF that will be active in the plant were unidirectional. pHIR-ACO was transformed into *A.tumafaciens* LBA4404: and this used to transform tomato cotyledons (*Lycopersicum esculentum* var Ailsa Craig). Plants were regenerated from callus.

Example 2.1

Identification of Transgenic Plants

DNA was extracted from single leaves and extracted as described previously. Plants containing the HIR-ACO T-DNA insert were identified by PCR using an internal TOM13 sense primer (5' GCTGGACTCAAGTTTCAAGCCAAAG 3'SEQ ID No 8) and a NOS 3'UTR (untranslated region) specific antisense primer (5'CCATCTCATAAATAACGTCATGC3' SEQ ID No 9)

Example 2.2

ACC-oxidase assays

ACC-oxidase activity was measured as the ability of plant tissue to convert exogenous 1-aminocyclopropane-1-1carboxylic acid (ACC) to ethylene. Small leaves were removed from shoots and wounded with a scalpel before being placed into a 2ml sealable vial, and left for 30minutes. The vials were then sealed and left for an hour at room temperature , after which the ethylene in the head space was measured by gas chromatography as described my Smith et al., 1986. Ethylene was also measured from wildtype, over-expressing (C12) and antisense down-regulated plant material.

47/4 20-04-2005

Printed: 22-04-2005 TIPAT 99910592

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: ZENECA LIMITED
(B) STREET: 15 STANHOPE GATE
(C) CITY: LONDON
(D) STATE: LONDON
(E) COUNTRY: UNITED KINGDOM
(F) POSTAL CODE (ZIP): W1Y 6LN
(G) TELEPHONE: 01344 414521
(H) TELEFAX: 01344 481112
(I) TELEX: 847556

(ii) TITLE OF INVENTION: GENE SILENCING

(iii) NUMBER OF SEQUENCES: 15

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3681 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(vii) IMMEDIATE SOURCE:
(B) CLONE: SEQ ID NO 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AAATTTGATA GATTCAGTTT TTATGTTTTT AGTGCTGATT ACAACATTGA AATTCTAAAT 60

TTAGAATTTA ATATTTATTA AATGTTAGTG CATTTATACA AATAACATAT TACATCTCAA 120

ATAATATTGA GTTTGTTAGA TTTTATTTGC CCTGATTTCT TATCATAAAT AGGTTTTCCT 180

TTTAGGAAAA GGTTTTGAAT TGACTATTCT TTTTTTGGTA GGAAAAAGTT TAGGACTCTA 240

TAAATAGAGG CATGTTCCTT CTAACTTAAT TAGCATTCAC AATGTAGTTT TAAGGGCTTT 300

GAGAGTTTTG GTTAGAGGGA GAATTTGTGA ACCTCTCATG TATTCCGAGT GAATTGGTTG 360

AGGTTGTTTC CCTCTGTATT TTGTACTCTC ATGTTTATAG TGGATTGCTC ATTTCCTTTG 420

TGGACGTAGG TCGATTGACC GAACCACGTT AAATTTTTGT GTCTTTTGGT ATATTTCCTG 480

TTCTTCTTAC TCGTGGTCTT TCGAGGTTTG CTTTGCTAGC TTCCGCGTTT ACACCTGCTT 540

20-04-2005

Printed:22-04-2005 TIPA 99910592

ATTTTCGGTC CTAACAAGTG GTATCAGAGC CAGATTCAAT AATGGAGTCA GGTGTAGTGG 600

TTCGATAATC GATGATTGAA CCAAGTTAGA AAGAGGTGTT CATCTTGACG GGTGTAGTTC 660

TAGCCGCAAC CTTTTTGACA GTAATGAAGA TTTTGATGGA GAAATTGTTT CAGAGAGGTT 720

CTCTGTGTTG AGACATAAAT TTTGTAAAGG AGATTATGGA GAGGAGAAGC AAGTTGTTGA 780

AGATTAAGTA AAGAAGGTGG ACAAATCTAT TTTGTCAGAA ATTCAGGCCA AGGGGGAGAT 840

TTGTTGGGTT TTATTTGCCC TGATTTTTTA CCATAAATAG GTTTTCCTTT AAGGAAAAGG 900

TTTTGAATTG ACTATTCTTT TTTTGGTAGG AAAAGGTTTA GGATTCTATA AATAGAGGCA 960

TGTTCCTTCT AACTTAATTA GCATTCACAA TGTAGTTTTA AGGGCTTTGA GAGTTTTGGT 1020

TAGAGGGAGA ATTTGTGAAC CTCTCATGTA TTCCGAGTGA ATTGGTTGAG GTTGTTTCCC 1080

TCTGTATTTT GTACTCTCAT GTTTATAGTG GATTGCTCAT TTCCTTTGTG GACGTAGGTC 1140

GATTGACCGA ACCACGTTAA ATCTTTGTGT CTTTTGGTAT ATTTCTCGTT GTCTTCTTAC 1200

TCGTGGTCTT TCGAGGTTTG CTTTGCTAGC TTCCGCGTTT ACACCTGCTT ATTTGCGGTC 1260

CTAACAGAGT TCGATGGGTT GAATCTATAA AAAGAAAAAT ATACTCGTGA TTCACGATTA 1320

TTTATATGAA AATATAATAA ATATTGAATT TCCTTTGCTA TTTCTTATGT TTACGTCTTT 1380

ATATTTCAAA TTATTCCACC AATACTGACA AGCCCTAGGC CATCTCTAGG AAATTCATAC 1440

AATTTTTTTT TTGTTGTTAA CTAGTTAAAT TGGCAGCCTT AAAGATTATT GTAAAATTCA 1500

AGGCAACTTC CTCAAGTACT ACAACTACAT TGTAACATCC CAGTCAAAGT GTCCTAAAAT 1560

TTTATAAAAT TTGACACATG AAACAATAGC ACAATAAATT TTAGTACTAT TGCAGCCATG 1620

GCCCATAAGC CATCATGTAT TATAGTCAAA ATGGGTCCTT TTCCAATTTG TCTTGATCCC 1680

AAAATCCCTT TGTAGGTAAG ATGGTTCAAC AAGGAACTAT GACTCTTAAG GTAGACTTGG 1740

ACTCATAGAC TTGTCATAAC TCATAAAGAC TTGGAATATA ATAATTATTC ATTTAAATTA 1800

TAATTCTCTA CTTTAATATC TTCTACTATA AATACCCTTT CAAAGCCTCA TTATTTGTAC 1860

ATCAAACATT GATATTCATC TCTTCAATCT TTTGTATTCA CATATTCTAT TTATTCAATA 1920

CACTTAGGAA AACACTTTAC CAAGAAATTA AGATGGAGAA CTTCCCAATT ATTAACTTGG 1980

AAAAGCTCAA TGGAGATGAG AGAGCCAACA CCATGGAAAT GATCAAAGAT GCTTGTGAGA 2040

ATTGGGGCTT CTTTGAGGTA ATCATAAATT ACATAAACAT ATTAATATGT TTGTTTCAAT 2100

TTATCAGTCA TACTTTTCTC TGTTTTAAAA TTAATGTCAC TTTCAATATT TAATAATTCG 2160

CATGACATGT TTATAACACA ACAAGATATA GGTTACATTT TGATACATTA TATATAACTT 2220

CTGTCACACG ACTCAAAAGT CTTTCTTAAT TTCTTGAATT CAATGATCGA TCAAACTAAG 2280

ACACGTAAAA TGAAACGGGG AATAGTAATT CTGTTTGCTT ATGTGATCAT TGTAGTTGGT 2340

GAACCATGGA ATTCCACATG AAGTAATGGA CACAGTAGAG AAAATGACAA AGGGACATTA 2400

CAAGAAGTGC ATGGAACAGA GGTTTAAGGA ACTAGTGGCA AGTAAGGGAC TTGAGGCTGT 2460

478 20-04-2005

TCAAGCTGAG GTTACTGATT TAGATTGGGA AAGCACTTTC TTCTTGCGCC ATCTTCCTAC 2520

TTCTAATATC TCTCAAGTAC CCGATCTTGA CGAAGAATAC AGGTACATAC ATGTGTCCTA 2580

CATATTGCGT ATATAATAAA TAAACACAAA ATTTAAGTTA TATACGCTGA CAGTATAACT 2640

AATTATAATG TTGTACCAAA TGATGCAGAG AGGTGATGAG AGATTTTGCT AAAAGATTGG 2700

AGAAATTGGC TGAGGAGTTA CTTGACTTAC TCTGTGAAAA TCTTGGACTT GAAAAAGGTT 2760

ACTTGAAAAA TGCCTTTTAT GGATCAAAAG GTCCCAACTT TGGTACTAAA GTTAGCAACT 2820

ATCCACCATG TCCTAAGCCC GATTTGATCA AGGGACTCCG CGCTCATACA GACGCAGGAG 2880

GCATCATACT TCTGTTCCAA GATGACAAAG TGAGTGGCCT TCAACTCCTC AAAGACGAGC 2940

AATGGATCGA TGTTCCTCCC ATGCGCCACT CTATTGTGGT TAACCTTGGT GACCAACTTG 3000

AGGTACAAGA TTCACTAAGT GTGTGTGTTT TTATCACTAT AACTTAGAAG TAGTAACTAA 3060

AAATGGTATT AATGAAATGT TATAAAAACA GGTGATCACT AACGGGAAGT ACAAGAGTGT 3120

GCTGCACAGA GTAATTGCAC AAACAGACGG GACACGAATG TCATTAGCCT CATTTTACAA 3180

TCCAGGAAGT GATGCAGTAA TATATCCAGC AAAAACTTTG GTTGAAAAAG AGGCAGAGGA 3240

AAGTACACAA GTGTATCCAA AGTTTGTGTT TGATGATTAC ATGAAGTTAT ATGCTGGACT 3300

CAAGTTTCAA GCCAAAGAGC CAAGATTTGA AGCAATGAAG GCAATGGAAA GTGATCCAAT 3360

TGCAAGTGCT TAGATCCCAA TTCAATTAAA AAAATTGGTG TTTGAAAAAT ATATTTAAAT 3420

ATAGCAATCT ATGTATACAC ATTATTTGCT CTTCTTATGT ATGGTAGAAT AAAGTTAGTA 3480

TTAAAAAAGA TTGTGATTTG CTGCATATGT ATCAAAAAGA GTCCTAATAT TTGTATCTAT 3540

AAATAAGGTG CCTTCTAGTG AAATTATACA AATAATAATT TGGAGTGTAT TGTTCTTTCT 3600

CATGTAATTT AACTTTTAAG TATCTTACTT TACAATATAC TGTTCACTTA TTGAACATAT 3660

TGAGTGATAT ATTGACTCAA T 3681

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CATTCATCTC TTCAATCTTT TG 22

(2) INFORMATION FOR SEQ ID NO: 3:

Printed: 22-04-2005 TIPA 99910592

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTTAATTTCT TGGTAAAGTG TTTTCC 26

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucliec acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATGGAGAACT TCCCAATTAT TAACTTGGAA AAG 33

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTAAGCACTT GCAATTGGAT CACTTTCCAT 30

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid

20-04-2005

(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTTTACCAAG AAGTGCACAT GGAGAACTTC CC 32

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 7

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GAATTGGGCC CTAAGCACTT GCAATTGG 28

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCTGGACTCA AGTTTCAAGC CAAAG 25

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

Received 22.04.2005 TIPA 99910592

(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 9

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCATCTCATA AATAACGTCA TGC 23

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1949 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PHIR-ACO SEQ ID NO 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACCGGTGAAT TCCCATGGAG TCAAAGATTC AAATAGAGGA CCTAACAGAA CTCGCCGTAA 60

AGACTGGCGA ACAGTTCATA CAGAGTCTCT TACGACTCAA TGACAAGAAG AAAATCTTCG 120

TCAACATGGT GGAGCACGAC ACGCTTGTCT ACTCCAAAAA TATCAAAGAT ACAGTCTCAG 180

AAGACCAAAG GGCAATTGAG ACTTTTCAAC AAAGGGTAAT ATCCGGAAAC CTCCTCGGAT 240

TCCATTGCCC AGCTATCTGT CACTTTATTG TGAAGATAGT GGAAAAGGAG GTGGCTCCTA 300

CAAATGCCAT CATTGCGATA AAGGAAAGGC CATCGTTGAA GATGCCTCTG CCGACAGTGG 360

TCCCAAAGAT GGACCCCCAC CCACGAGGAG CATCGTGGAA AAAGAAGACG TTCCAACCAC 420

GTCTTCAAAG CAAGTGGATT GATGTGATAT CTCCACTGAC GTAAGGGATG ACGCACAATC 480

CCACTATCCT TCGCAAGACC CTTCCTCTAT ATAAGGAAGT TCATTTCATT TGGAGAGGAC 540

AGGGTACCGC GGCACGGCCA GCCACGCCGC TGAGCCCGCA GTTTCTCGAG TTTCTGCGGG 600

CTCAGCGGCG TGGCTGGCCG TGCCGCCCAT GGGCGGCGGG GCTGCAGGAA TTCGATATCA 660

AGCTTATCGA TACCGTCGAC CTCGAGGGGG GGCCCGGTAC CGGATCCCCT GCACATGGAG 720

AACTTCCCAA TTATTAACTT GGAAAAGCTC AATGGAGATG AGAGAGCCAA CACCATGGAA 780

ATGATCAAAG ATGCTTGTGA GAATTGGGGC TTCTTTGAGT TGGTGAACCA TGGAATTCCA 840

CATGAAGTAA TGGACACAGT AGAGAAAATG ACAAAGGGAC ATTACAAGAA GTGCATGGAA 900

CAGAGGTTTA AGGAACTAGT GGCAAGTAAG GGACTTGAGG CTGTTCAAGC TGAGGTTACT 960

GATTTAGATT GGGAAAGCAC TTTCTTCTTG CGCCATCTTC CTACTTCTAA TATCTCTCAA 1020

480 20-04-2005

GTACCCGATC TTGACGAAGA ATACAGAGAG GTGATGAGAG ATTTTGCTAA AAGATTGGAG 1080

AAATTGGCTG AGGAGTTACT TGACTTACTC TGTGAAAATC TTGGACTTGA AAAAGGTTAC 1140

TTGAAAAATG CCTTTTATGG ATCAAAAGGT CCCAACTTTG GTACTAAAGT TAGCAACTAT 1200

CCACCATGTC CTAAGCCCGA TTTGATCAAG GGACTCCGCG CTCATACAGA CGCAGGAGGC 1260

ATCATACTTC TGTTCCAAGA TGACAAAGTG AGTGGCCTTC AACTCCTCAA AGACGAGCAA 1320

TGGATCGATG TTCCTCCCAT GCGCCACTCT ATTGTGGTTA ACCTTGGTGA CCAACTTGAG 1380

GTGATCACTA ACGGGAAGTA CAAGAGTGTG CTGCACAGAG TAATTGCACA AACAGACGGG 1440

ACACGAATGT CATTAGCCTC ATTTTACAAT CCAGGAAGTG ATGCAGTAAT ATATCCAGCA 1500

AAAACTTTGG TTGAAAAAGA GGCAGAGGAA AGTACACAAG TGTATCCAAA GTTTGTGTTT 1560

GATGATTACA TGAAGTTATA TGCTGGACTC AAGTTTCAAG CCAAAGAGCC AAGATTTGAA 1620

GCAATGAAGG CAATGGAAAG TGATCCAATT GCAAGTGCTT AGGGGAGCCT GGGCCCCTGC 1680

AGGTCGTTCA AACATTTGGC AATAAAGTTT CTTAAGATTG AATCCTGTTG CCGGTCTTGC 1740

GATGATTATC ATATAATTTC TGTTGAATTA CGAATTGCAT GTAATAATTA ACATGTAATG 1800

CATGACGTTA TTTATGAGAT GGGTTTTTAT GATTAGAGTC CCGCAATTAT ACATTTAATA 1860

CGCGATAGAA AACAAAATAT AGCGCGCAAA CTACCATAAA TTATCGCGCG CGGTGTCATC 1920

TATGTTACTA GATCGGGAAG CTTACCGGT 1949

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: SYNTHERTIC INVERTED REPEAT SEQ ID NO 11

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCGGCACGGC CAGCCACGCC GCTGAGCCCG CAGTTTCTCG AGTTTCTGCG GGCTCAGCGG 60

CGTGGCTGGC CGTGCCGC 78

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

Printed:22-04-2005 TIPA 99910592

(A) DESCRIPTION: /desc = "PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 12

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GCGGGTACCG CGGCACGGCC AGCCACGCCG CTGAGCCCGC AGTTTCTCGA GGATGGGTTG 60

GCTCCATGGG CGGCG 75

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 13

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CGCCGCCCAT GGAGCCAACC CATCCTCGAG AAACTGCGGG CTCAGCGGCG TGGCTGGCCG 60

TGCCGCGGTA CCCGC 75

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 14

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GGGGCGCCGC TCGAGTTTCT GCGGGCTCAG CGGCGTGGCT GGCCGTGCCG CCCATGGCGC 60

ATCGGG 66

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs

20-04-2005

(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR PRIMER"

(vii) IMMEDIATE SOURCE:
(B) CLONE: PCR PRIMER SEQ ID NO 15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCCTAGGCGC CATGGGCGGC ACGGCCAGCC ACGCCGCTGA GCCCGCAGAA ACTCGAGCGG 60

CGCCCC 66

CLAIMS

1. A vector for enhancing the inhibition of a selected target gene within an organism, comprising a gene silencing vector characterised in that the said gene silencing vector includes a inverted repeat of all or part of a polynucleotide region within the vector.

2. A vector as claimed in claim 1, in which the inverted repeat sequence is a synthetic polynucleotide sequence and its inverted repeat sequence.

3. A vector as claimed in claim 1, in which the inverted repeat sequence is an inverted repeat of all or part of the said gene silencing vector.

4. A vector as claimed in claim 3, in which the inverted repeat sequence is an inverted repeat of the 5'-untranslated region of the gene silencing vector.

5. A method as claimed in any of claims 1 to 4, in which the inverted repeat is separated from the polynucleotide region by a sequence of nucleotides.

6. A method of controlling the expression of a DNA sequence in a target organism, comprising inserting into the genome of said organism an enhanced gene silencing vector as claimed in any of claims 1 to 4.

7. A vector for enhanced gene silencing comprising in sequence a promoter region, a 5'-untranslated region, a transcribable DNA sequence and a 3'-untranslated region containing a polyadenylation signal, characterised in that the said construct includes an inverted repeat of a region of said construct.

8. A vector as claimed in claim 7 in which the inverted repeat is a fragment of the 5'-untranslated region of the said construct.

9. A vector as claimed in claim 7 or claim 8, in which the inverted repeat is separated from the selected fragment by a sequence of nucleotides acting as a spacer.

10. A vector as claimed in claim 7 or 8 or 9, in which the construct includes a double copy of the inverted repeat.

11. A vector as claimed in any of claims 7 to 10, in which the vector two tandem copies of the inverted repeat.

12. A DNA construct for the inhibition of gene expression comprising in sequence a promoter region, a 5'-untranslated region, a transcribable DNA sequence and a 3'-untranslated region containing a polyadenylation signal, characterised in that the said 5'-untranslated region is contiguous with a pair of tandem inverted repeats of said 5'-untranslated region.

Printed 22.04.2005

TIPAT

99910592

FIGURE 1A

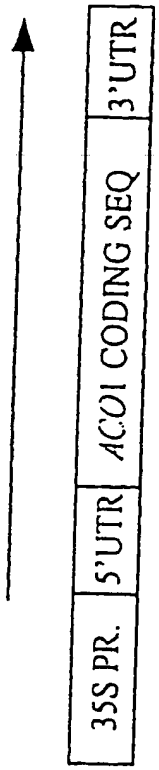
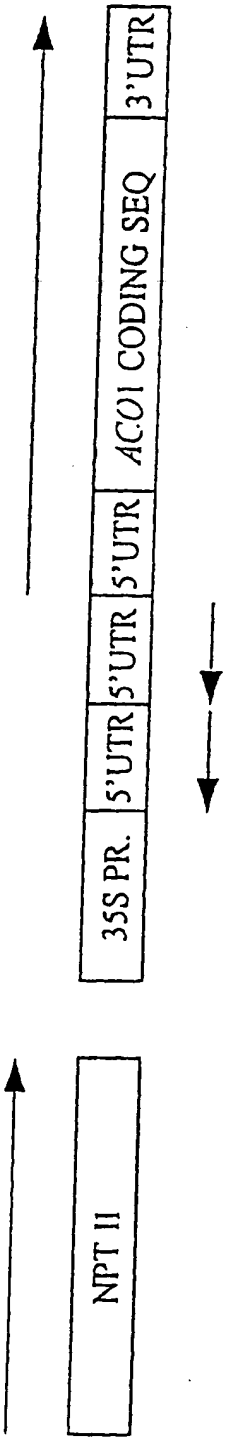


FIGURE 1B



486

20-04-2005

FIGURE 2

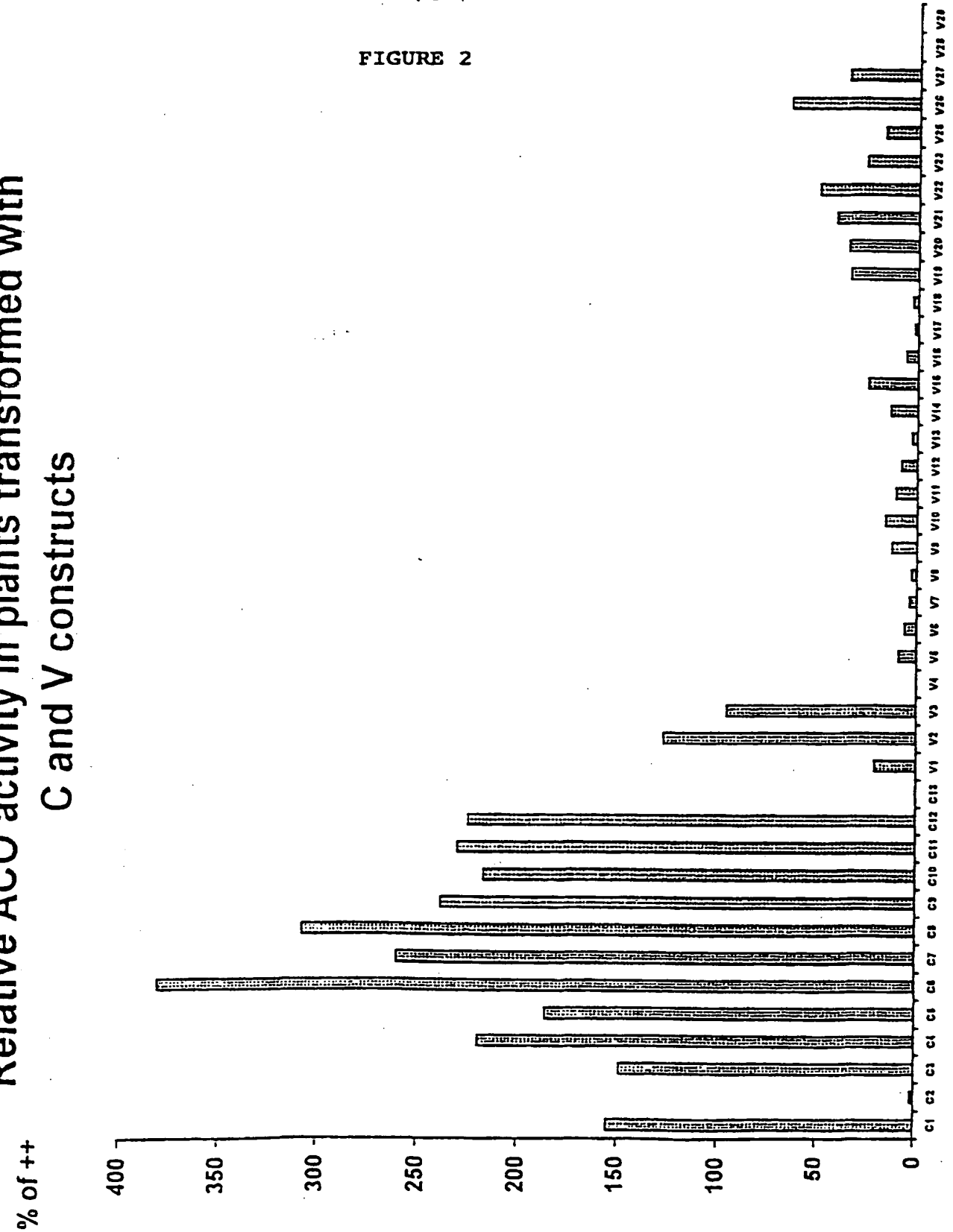
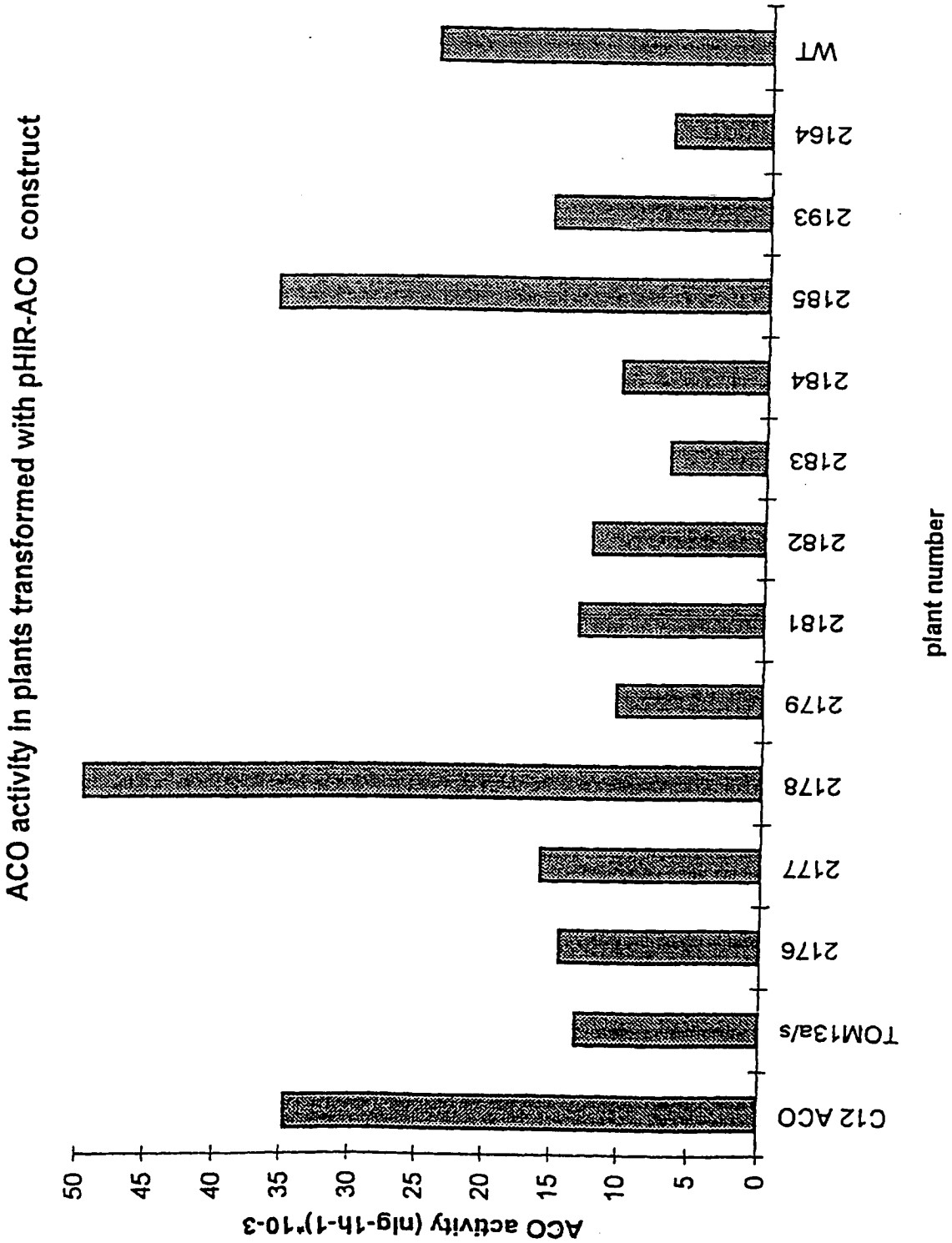

Relative ACO activity in plants transformed with C and V constructs
% of ++
400
350
300
250
200
150
100
50
0
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 V17 V18 V19 V20 V21 V22 V23 V24 V25 V26 V27 V28

FIGURE 3


ACO activity in plants transformed with pHIR-ACO construct
ACO activity (nlg-1h-1)*10-3
0
5
10
15
20
25
30
35
40
45
50
C12 ACO
TOM13a/s
2176
2177
2178
2179
2181
2182
2183
2184
2185
2193
2164
WT
plant number

Printed 22-04-2005

TIPAT

99910592

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01450

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/63 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DORER D. AND HENIKOFF S.: "Expansions of transgene repeats cause heterochromatin formation and gene silencing in Drosophila" CELL, vol. 77, no. 7, 1 July 1994, pages 993-1002, XP002075449 * see the whole document, esp. discussion, figure 5 *	1,3,7
A	ASSAAD F. ET AL.: "Epigenetic repeat-induced gene silencing (RIGS) in Arabidopsis" PLANT MOLECULAR BIOLOGY, vol. 22, no. 6, September 1993, pages 1067-1085, XP002075450 * See the whole document, esp. p.1081 l. col. 1.30 - r. col. 1.3 *	1-12

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 August 1998	14/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

Form PCT/ISA/210 (second sheet) (July 1992)

20-04-2005

Printed: 22-04-2005 TIPAT 999 0592

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 98/01450

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TEN LOHUIS M. ET AL.: "A repetitive DNA fragment carrying a hot spot for de novo DNA methylation enhances expression variegation in tobacco and petunia" PLANT JOURNAL, vol. 8, no. 6, December 1995, pages 919-932, XP002075451 cited in the application see the whole document	1-12
A	WO 97 01952 A (DNA PLANT TECHN CORP) 23 January 1997 * see esp. p.13-15 *	1-12
A	WO 93 23551 A (SEYMOUR GRAHAM BARRON ;TUCKER GREGORY ALAN (GB); GRIERSON DONALD () 25 November 1993 see the whole document	1-12
A	GRIERSON, DON: "Silent genes and everlasting fruits and vegetables" NAT. BIOTECHNOL. (1996), 14(7), 828-829 CODEN: NABIF9;ISSN: 1087-0156, XP002075452 see the whole document	1-12
A	BLUME B ET AL: "Identification of transposon-like elements in non-coding regions of tomato ACC oxidase genes." MOLECULAR AND GENERAL GENETICS, (1997 APR 16) 254 (3) 297-303. JOURNAL CODE: NGP. ISSN: 0026-8925., XP002075453 see the whole document	1-12
A	HAMILTON, A. J. ET AL: "Post-transcriptional gene-silencing in tomato" MECH. APPL. GENE SILENCING, 'EASTER SCH. AGRIC. SCI.!, 57TH (1996), MEETING DATE 1995, 105-117. EDITOR(S): GRIERSON, DONALD;LYCETT, GRANTLEY W.; TUCKER, GREGORY A. PUBLISHER: NOTTINGHAM UNIVERSITY PRESS, NOTTINGHAM, UK. CODEN: 63NBAT, XP002075454 see the whole document	1-12
T	STAM, M. ET AL: "Post-transcriptional silencing of chalcone synthase in Petunia by inverted transgene repeats" PLANT J., (19970700) VOL. 12, NO. 1, PP. 63-82. ISSN: 0960-7412., XP002075455 see the whole document	1-12

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

20-04-2005

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 98/01450

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9701952 A	23-01-1997	AU 6402996 A	05-02-1997
		EP 0837624 A	29-04-1998
WO 9323551 A	25-11-1993	AU 4079493 A	13-12-1993
		EP 0644942 A	29-03-1995
		ZA 9303361 A	23-09-1994

Form PCT/ISA/210 (patent family annex) (July 1992)

20-04-2005

TIPAT

The Patent Office

99910 32

PCT/GB 9 8 / 0 1 4 5 0

2 0 May 1998

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP9 1RH

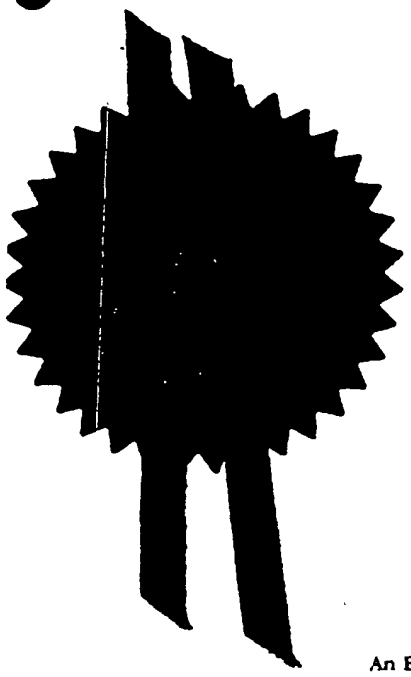
REC'D 3 0 JUN 1998
WIPO PCT

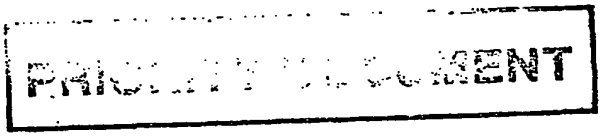
I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.





Signed

Dated 17 June 1998

20-04-2005

Printed: 22-04-2005

TIPA1

99910592

Patents Form 1/77

Act 1977

The Patent Office

1/77

THE PATENT OFFICE 21 MAY 1997 RECEIVED BY HAND

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference

PPD 50223/GB/P

21 MAY 1997

2. Patent application number
(The Patent Office will fill in this part)

9710475.6

ZPHT3Y E276402-1 000055
P01/7700 25.00 - 9710475.6

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ZENECA Limited
15 Stanhope Gate
London W1Y 6LN
UNITED KINGDOM

Patents ADP number *(if you know it)*

625400700

If the applicant is a corporate body, give the country/state of its incorporation

UNITED KINGDOM

4. Title of the invention

GENE SILENCING

Name of your agent *(If you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Frank M Huskisson
Intellectual Property Department
ZENECA Agrochemicals
Jealott's Hill Research Station
PO BOX 3538 Bracknell
Berkshire RG42 6YA
UNITED KINGDOM

Patents ADP number *(if you know it)*

~~039690600002~~

If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or

b) there is an inventor who is not named as an applicant, or

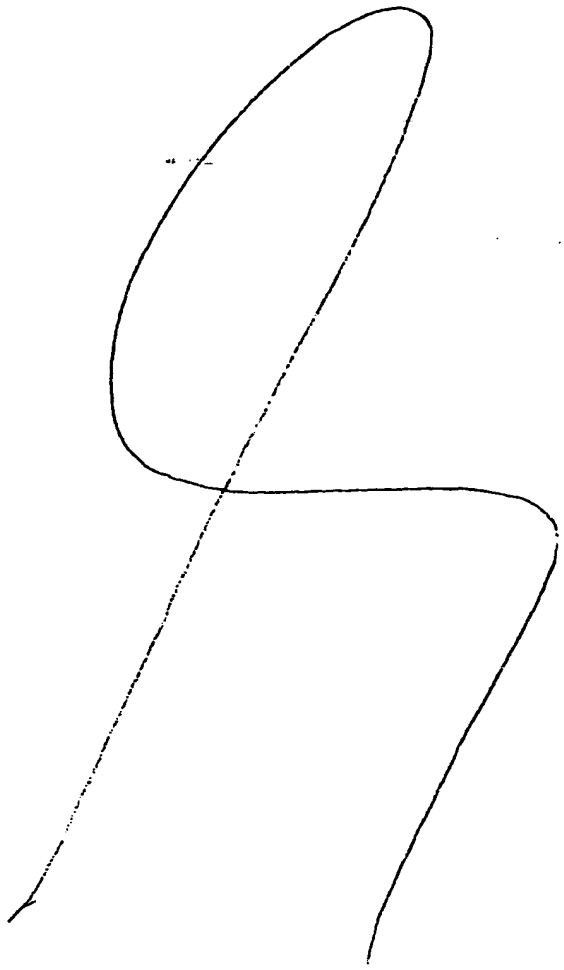
c) any named applicant is a corporate body.

See note (d))

494

20-04-2005

Faxed 22-04-2005 TIPA 999T0592



495 20-04-2005

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	20
Claim(s)	1
Abstract	–
Drawing(s)	2 + 2

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	1
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	Hard copy of Sequence Listing together with Diskette containing same

I/We request the grant of a patent on the basis of this application.

ZENECA Limited

Signature V.K.Mallipeddi Date 21/5/97

Authorised Signatory

Name and daytime telephone number of person to contact in the United Kingdom

VIJAYA KUMARI MALLIPEDDI 01344 414365

MONIQUE ADAMS 01344 414079

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

Write your answers in capital letters using black ink or you may type them.

If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

If you have answered 'Yes' Patents Form 7/77 will need to be filed.

Once you have filled in the form you must remember to sign and date it.

For details of the fee and ways to pay please contact the Patent Office.

Printed: 22-04-2005
TIPA
99910592

GENE SILENCING

This invention relates to the control of gene expression, more particularly to the inhibition of expression, commonly referred to as "gene silencing".

Three principal methods for the modulation of gene expression are known. These are referred to in the art as "antisense downregulation" and "sense downregulation", also, referred to as "cosuppression". Both of these methods lead to an inhibition of expression of the target gene. Overexpression of a target gene is achieved by insertion of one or more than one extra copies of the selected gene. Other methods which are used involve modification of the genetic control elements, the promoter and control sequences, to achieve greater or lesser expression of an inserted gene.

In antisense downregulation, a DNA which is complementary to all or part of the target gene is inserted into the genome in reverse orientation. While the mechanism has not been fully elucidated, one theory is that transcription of such an antisense gene produces mRNA which is complementary in sequence to the mRNA product transcribed from the endogenous gene: that antisense mRNA then binds with the naturally produced "sense" mRNA to form a duplex which inhibits translation of the natural mRNA to protein. It is not necessary that the inserted antisense gene be equal in length to the endogenous gene sequence: a fragment is sufficient. The size of the fragment does not appear to be particularly important. Fragments as small as 40 or so nucleotides have been reported to be effective. Generally somewhere in the region of 50 nucleotides is accepted as sufficient to obtain the inhibitory effect. However, it has to be said that fewer nucleotides may very well work: a greater number, up to the equivalent of full length, will certainly work. It is usual simply to use a fragment length for which there is a convenient restriction enzyme cleavage site somewhere downstream of fifty nucleotides. The fact that only a fragment of the gene is required means that not all of the gene need be sequenced. It also means that commonly a cDNA will suffice, obviating the need to isolate the full genomic sequence.

4196
20-04-2005

The antisense fragment does not have to be precisely the same as the endogenous complementary strand of the target gene. There simply has to be sufficient sequence similarity to achieve inhibition of the target gene. This is an important feature of antisense technology as it permits the use of a sequence which has been derived from one plant species to be effective in another and obviates the need to construct antisense vectors for each individual species of interest. Although sequences isolated from one species may be effective in another, it is not infrequent to find exceptions where the degree of sequence similarity between one species and the other is insufficient for the effect to be obtained. In such cases, it may be necessary to isolate the species-specific homologue.

Antisense downregulation technology is well-established in the art. It is the subject of several textbooks and many hundreds of journal publications. The principal patent reference is European Patent No. 240,208 in the name of Calgene Inc. There is no reason to doubt the operability of antisense technology. It is well-established, used routinely in laboratories around the world and products in which it is used are on the market.

Both overexpression and downregulation are achieved by "sense" technology. If a full length copy of the target gene is inserted into the genome then a range of phenotypes is obtained, some overexpressing the target gene, some underexpressing. A population of plants produced by this method may then be screened and individual phenotypes isolated. A similarity with antisense is that the inserted sequence need not be a full length copy. The principal patent reference on cosuppression is European Patent 465,572 in the name of DNA Plant Technology Inc. There is no reason to doubt the operability of sense/cosuppression technology. It is well- established, used routinely in laboratories around the world and products in which it is used are on the market.

Sense and antisense gene regulation is reviewed by Bird and Ray in Biotechnology and Genetic Engineering Reviews 9: 207-227 (1991). The use of these techniques to control selected genes in tomato has been descibed by Gray et.al., Plant Molecular Biology, 19: 69-87 (1992).

22-04-2005 TIPA 99910592

Gene control by any of the methods described requires insertion of the sense or antisense sequence, under control of appropriate promoters and termination sequences containing polyadenylation signals, into the genome of the target plant species by transformation, followed by regeneration of the transformants into whole plants. It is probably fair to say that transformation methods exist for most plant species or can be obtained by adaptation of available methods.

The most widely used method is *Agrobacterium*- mediated transformation, mainly for dicotyledonous species. This is the best known, most widely studied and, therefore, best understood of all transformation methods. The rhizobacterium *Agrobacterium tumefaciens*, or the related *Agrobacterium rhizogenes*, contain certain plasmids which, in nature, cause the formation of disease symptoms, crown gall or hairy root tumours, in plants which are infected by the bacterium. Part of the mechanism employed by *Agrobacterium* in pathogenesis is that a section of plasmid DNA which is bounded by right and left border regions is transferred stably into the genome of the infected plant. Therefore, if foreign DNA is inserted into the so-called "transfer" region (T-region) in substitution for the genes normally present therein, that foreign gene will be transferred into the plant genome. There are many hundreds of references in the journal literature, in textbooks and in patents and the methodology is well-established.

Various methods for the direct insertion of DNA into the nucleus of monocot cells are known.

In the ballistic method, microparticles of dense material, usually gold or tungsten, are fired at high velocity at the target cells where they penetrate the cells, opening an aperture in the cell wall through which DNA may enter. The DNA may be coated on to the microparticles or may be added to the culture medium.

In microinjection, the DNA is inserted by injection into individual cells via an ultrafine hollow needle.

495 20-04-2005

Printed: 22-04-2005 TIPAT 99910592

Another method, applicable to both monocots and dicots, involves creating a suspension of the target cells in a liquid, adding microscopic needle-like material, such as silicon carbide or silicon nitride "whiskers", and agitating so that the cells and whiskers collide and DNA present in the liquid enters the cell.

In summary, then, the requirements for both sense and antisense technology are known and the methods by which the required sequences may be introduced are known.

An object of the present invention is to provide a mechanism for the control of gene expression.

According to the present invention there is provided a method of controlling the expression of a DNA sequence in a target organism, comprising inserting into the genome of said organism a gene construct comprising in sequence a promoter region, a 5'-untranslated region, a transcribable DNA sequence and a 3'-untranslated region containing a polyadenylation signal, characterised in that the said construct includes an inverted repeat of a fragment of said construct.

The inverted repeat may be of a fragment of the 5'-untranslated region of the said construct. Preferably also the inverted repeat is separated from the selected fragment by a sequences of nucleotides acting as a separator. Preferably the construct includes a double copy of the inverted repeat. More preferably the construct has a selected fragment fused to two copies in tandem of the inverted repeat.

In a preferred embodiment of the invention a gene construct comprises in sequence a promoter region, a 5'-untranslated region, a transcribable DNA sequence and a 3'-untranslated region containing a polyadenylation signal, characterised in that the said 5'-untranslated region is followed immediately by a pair of tandem inverted repeats of said 5'-untranslated region..

In general terms, the invention is based on the surprising discovery that the level of expression inhibition obtained by a conventional downregulation gene cassette may be

199 20-04-2005

Received: 22-04-2005 TIPA 99910592

enormously increased by including in the construct an inverted repeat of a section of the DNA sequence of the construct. The mechanism by which such a construct of the invention may work is unknown.

Without wishing to be bound by any particular theory of how the it may work, the following is a discussion of our invention. 96% of tomato plants transformed with an ACC-oxidase sense gene containing two additional, upstream inverted copies of its 5' untranslated region, exhibited substantially reduced ACC-oxidase activity compared to wild type plants. Only 15% of plants transformed with a similar construct, without the inverted repeat, had reduced ACC-oxidase activity. Both populations had similar average numbers of transgenes per plant. Treatment of tomato leaves with cycloheximide caused a strong, reproducible increase in the abundance of ACC-oxidase transcripts and was used in the study of suppression by ACC-oxidase sense transgenes in preference to wound induction used in previous studies. The relative abundance of unprocessed and processed ACC-oxidase transcripts in suppressed and non-suppressed plants was assayed by ribonuclease protection assays, providing an indirect measure of transcription and mRNA accumulation which did not rely upon assaying isolated nuclei. This analysis indicated that the suppression of ACO1 gene expression was mainly post-transcriptional. Using the same type of RPA assay similar results were obtained from plants containing suppressing polygalactronase-sense or ACO-antisense transgenes.

There are now numerous examples of the inactivation of homologous sequences in plants. The term "homology dependent gene silencing" (HDGS) best describes all of these although it should be noted that in most examples the "silencing" is not complete and a low level of gene expression remains. Throughout this paper we will use the classification most-recently outlined by Matzke and Matzke, Plant Physiol. 107: 679-685 (1995) in which different examples of HDGS were divided into three main groups; cis-inactivation, trans-inactivation, and sense-suppression. Down regulation by antisense genes bears many similarities to the last of these and has been suggested to operated by the same mechanism (Grierson et al, Trends Biotechnol. 9: 122-123 (1991)). Both sense and antisense transgenes have been widely used to reduce the expression of homologous endogenous genes in plants.

20-04-2005

Although the underlying mechanisms of HDGS remain obscure, this technology has found numerous applications not only in fundamental research but also in commercial biotechnology ventures and new food products are already on the market.

At present , obtaining a large number of strongly suppressed, transgenic lines is more a matter of luck than judgement. A positive correlation between the presence of repeated transgene sequences and the incidence of HDGS has been noted. However single locus-transgene insertions associated with HDGS have also been reported.

There is an emerging consensus that different examples of HDGS can be classified on the basis of whether or not the transcription of the target gene is affected. Examples of transcriptional suppression have been described. Where the homology between interacting genes resides within transcribed sequences, HDGS has been shown to be a post-transcriptional effect. Despite this apparently precise demarcation, several similarities exist between some examples in the two different categories. These include variegated patterns of silencing, increased methylation of genes participating in silencing and the frequent observation that silencing loci contain repeated sequences.

Although transcriptional silencing must occur in the nucleus, post-transcriptional silencing might occur in either or both the nucleus or cytoplasm. There is evidence that the abundance of processed, nuclear RNA of silenced genes was unaffected and suggested an effect upon transport into or degradation within the cytoplasm. More compelling evidence that post-transcriptional HDGS occurs outside the nucleus is the relationship between gene silencing involving nuclear transgenes and resistance to cytoplamically replicating RNA viruses. Transgenic plants containing transgenes that suppress the activity of other transgenes (e.g. GUS) or endogenous genes (e.g. PG) are also resistant to RNA viruses which have been engineered to include sequences from those genes. Nevertheless, nuclear features such as transgene methylation and complexity of transgene loci were found positively to correlate with virus resistance. In almost all instances of HDGS, the source of the silencing is nuclear (even if the manifestation is cytoplasmic). However, silencing of a nuclear gene by a cytoplasmic element has been demonstrated by the suppression of phytoene desaturase in plants infected by a recombinant virus containing sequences from that gene.

Filed: 22-04-2005

TIPA

99910592

Although, there are now numerous examples of post-transcriptional suppression of plant genes by HDGS, as yet, there is no information as to whether the increased turnover of pre-mRNA is related to or distinct from other cellular, RNA turnover processes. Degradation of RNA in plants is poorly understood but there is evidence that translation is involved. For example, the very short half lives (around 10 minutes) of small auxin up RNAs (SAURS) can be markedly prolonged by treatment with cycloheximide.

This invention gives a striking increase in the frequency of HDGS following the inclusion of a short repeated region within a transgene. Expression of the target gene encoding the terminal ethylene biosynthetic enzyme ACC-oxidase, in tomato was suppressed by such constructs mainly post-transcriptionally. This was shown to be true for other examples of sense and antisense suppression in tomato. Cycloheximide was found to be a potent and reliable inducer of ACO gene expression but did not ameliorate the silencing.

The invention will now be described, by way of illustration, in the following Examples and with reference to the accompanying Figures.

Example 1

Construct V (Figure 1) was made in the following manner: 79 base pairs of the 5' untranslated region of the tomato ACO1 cDNA was amplified by PCR and two copies were ligated in tandem in the reverse orientation immediately upstream of the ACO1 cDNA which contains its own polyadenylation signal in its 3' untranslated region (construct C). Both were ligated downstream of the CaMV 35S promoter and then transferred to the binary vector, Bin19. Figure 1 shows the basic details of constructs C and V. These were used to transform tomato plants (Ailsa Craig) by *Agrobacterium* mediated DNA transfer. 13 and 28 individual kanamycin resistant calli were obtained with constructs C and V respectively and these were regenerated into plants.

The nucleotide sequence of the promoter and 5' untranslated region of the ACO1 gene is given as SEQ ID NO 1 hereinafter. The 79bp referred to above begins at base number 1874 and stops at the base immediately preceding the translation start codon (ATG) at number 1952.

5002

20-04-2005

Example 2

To screen the population for any effects on ACO gene expression, relative ACO activity was measured from untransformed and transformed plants. The production of ethylene from leaf discs supplemented with the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, was measured at least three times from each plant. The cutting of the discs by a cork borer wounds the leaves and stimulates the expression of the ACO1 gene. ACC-oxidase activity in both types of transgenic plant relative to wild type values are shown in Figure 2. There was a dramatic difference in ACO activity between the two populations, with plants containing the inverted repeat (V line) showing very strong suppression. The majority (11 out of 13) of plants of the C line did not show suppression of ACO activity but overexpression, compared to wild-type plants, as would be expected since this construct contained a translatable ACO1 coding sequence.

To test for the presence of the transgenic ACO sequence, DNA from the plants was analysed by PCR using two oligos homologous to and complementary with the beginning and end respectively of the ACO1 coding sequence. This combination co-amplifies 1500 bp of the endogenous ACO1 gene (which acts as an internal positive control) and the ACO1 sense transgene as a 1000 bp fragment (since it was derived from a cDNA and so has no introns). The amplified region does not include the repeated region of the V-type transgene. The two fragments were separated by gel electrophoresis and detected by staining with ethidium bromide. This showed the presence of the transgene in all plants of the C line and all plants of the V line except one (V2) which also had no reduced ACC-oxidase activity (Figure 2).

Example 3

It was considered possible that the repeated region in the transgene might have affected the number of transgenes which integrated into the genome and that this was the actual source of high frequency silencing. The PCR assay described above can be used to estimate the transgene copy number if the following assumptions are made:

1) that in any transgenic plants there was no variation in the number of endogenous ACO1 genes per genome;

22-04-2005
TIPAT
99910592

2) that the amplification efficiency ratio (endogenous ACO1 DNA: transgenic ACO1 DNA) is constant;

3) the reaction is sampled at low DNA concentration to minimise product reannealing. Since we were only concerned with estimating the number of transgenes in the two lines relative to each other and not absolute quantification of transgene copy number, we did not employ synthetic combinations of "transgene" and "endogenous gene" DNA as standards.

After 20 cycles of amplification, gel-electrophoresis, Southern blotting, and hybridisation with a radioactively labelled ACO1 cDNA, the signal from endogenous and transgenic ACO1 DNA was visualised and quantified by phoshorimaging. The average transgene: endogenous gene ratio for the C line was 0.96 and for the V line 1.08 indicating that the repeat region in the V construct does not cause more T-DNAs to integrate during transformation.

Example 4

ACO1 mRNA increased in abundance following wounding and/or treatment of leaves with cycloheximide but accumulation was approximately five times greater after treatment with cycloheximide than after mechanical wounding which we have previously used as a stimulus. Wounding of cycloheximide treated leaves failed to elicit a further increase in ACO1 mRNA amount. We found cycloheximide to be a more reproducible inducer of ACO1 mRNA accumulation than mechanical wounding and so have used it in preference to the latter in this study. No further increase in the abundance of ACO1 mRNA was observed when the concentration of cycloheximide was increased from 50 to 250 ug/ml (date not shown).

Example 5

The 5' end of ACO1 mRNA extracted from plants is heterogeneous but consists of two major species which differ by 2 bases. The 5' untranslated region (both the sense and duplicated antisense sequences) in both of the constructs (C and V) was made approximately 10 base pairs shorter than those of the endogenous gene. This allowed the discrimination of

20-04-2005

endogenous gene and transgene-derived transcripts by ribonuclease protection assays using a probe transcribed from a genomic ACO1 sequence which extended from the start of the 3' end of the 5' untranslated region to a AccI site, in the promoter of ACO1, 222 bases upstream. In RNA from wild type leaves, there were several bands which may arise from distinct RNA species or from breaking of RNA duplexes during digestion. Some of the bands seem more susceptible to the effects of antisense suppression than others (although the general trend is still suppression).

In leaves from lines V4, V11 and V28 (all <10% ACO activity), there was extensive co-suppression of the endogenous transcripts (relative to wild-type) and the transgene transcripts (relative to those from a control transgene (line C1). V4, V11 and V28 all exhibited greater suppression than the homozygous ACO-antisense line (Hamilton et.al. Nature 346, 284-287(1990)).

The use of the protein synthesis inhibitor cycloheximide as a stimulant of ACO1 RNA accumulation did not obviously alleviate the suppression of this RNA by the sense transgenes in lines V4, V11 or V28.

Although the endogenous genes transcript is unquestionably suppressed, it is possible that the inverted repeat within the 5' end of the V transgene transcript excludes the probe and causes the signal from the transgenic RNA to be underrepresented. This seems unlikely for the following reason. When a probe that was not excluded by the inverted repeat was used to analyse RNA from the V line, the mRNA signal (which, using this probe, is actually the sum of the endogenous and the transgenic RNAs) was still much less than in the wild type. The data shows that in the absence of silencing, the abundance of the endogenous and transgenic RNAs are comparable.

Example 6

We chose to measure the abundance of unprocessed transcripts in total RNA extracts as a indirect measurement of transcription whilst simultaneously measuring the amount of processed mRNA. This was achieved using RNA probes transcribed from genomic sequences spanning introns in ribonuclease protection assays. Since the RNA analysed was from leaves

20-04-2005 TIPAT 99910592

frozen in liquid nitrogen and then extracted in strongly protein-denaturing conditions (phenol and detergent) there should have been little opportunity for any resetting of transcription during the process There was a greater abundance of mRNA following treatment with cycloheximide although the total amount of mRNA in the ACO-AS plants was reduced. In the ACO-sense line, V11, there was little or no increase in the mRNA signal. It is likely that this mRNA signal is mainly from the transgene which is transcribed by the 35S promoter which is not cycloheximide inducible. In contrast, the abundance of the primary transcript in all RNA samples increased following cycloheximide treatment. This RNA species originates only from the endogenous ACO1 gene since the transgene has no introns. In all cases the suppressing transgene had little or no effect upon the abundance of the primary transcript.

Example 7

Cycloheximide strongly stimulated the accumulation of both the ACO1 primary transcript and mature mRNA. Quantification of the signal from primary transcripts and mature ACO1 RNA in wild type leaves before and after treatment with cycloheximide showed that there was a 6 fold increase in the abundance of unprocessed ACO1 RNA but a 13 fold increase in the amount of processed ACO1 RNA. The abundance of transgenic ACO1 RNA (transcribed from the 35S promoter) in the C line also rose upon treatment with cycloheximide.

Example 8

Two tandemly linked copies of the 5'UTR (each unit = 79bp; 74.7% (A+T)) were litigated in the inverted orientation between the CaMV 35S promoter and an almost full length ACO1 cDNA (Figure 1). Either unit of this direct repeat has the capacity to form a large cruciform structure with the 5'untranslated region immediately downstream. After *Agrobacterium*-mediated transformation with this construct, 26 out of 28 plants recovered from tissue culture exhibited suppressed ACO activity. A much lower frequency (2/15) of suppression was observed with a control construct which lacked the duplicated 5'UTR but was otherwise the same.

506 20-04-2005

Printed 22-04-2005 TIPA1 99910592

More transgenic plants were obtained with the V construct than with the control construct (as well as exhibiting the high HDGS frequency). It is likely that this is a direct result of reduced ethylene synthesis as a result of ACO gene suppression. Previous results have shown that greatly improved callus regeneration could be achieved after transformation with constructs which contained an ACO-antisense gene.

Of the two plants transformed with the repeat construct that showed no suppression, one, V2, may have had a truncated T-DNA or be an untransformed escape since the transgenic ACO1 sequence could not be amplified. Since the repeat contained DNA sequences already in the gene, it seems unlikely that it is this sequence per se which elicits the effect upon gene silencing. It is much more likely that it is the structure of the repeat DNA (or the transcribed RNA) which is the source of the high frequency of silencing observed. The repeat within the V construct was similar to that with the control construct

Most instances of HDGS are associated with complex transgenic loci that contain repeats or whole or part T-DNAs rather than simple single insertions but it is not known whether this is a primary determinant of suppression or an indirect effect. There are examples where apparently single transgenes are associated with gene silencing but these are in the minority and in at least some of these examples the T-DNAs contain internal repeats. The data presented here suggest that deliberate introduction of small repeats in a transgene can increase the number of transgenic lines in which homologous genes have been suppressed to almost 100%. Sense suppression could be obtained with the control construct but at a much lower frequency. The deliberate introduction of repetitive DNA into a transgene may substitute for a requirement for the insertion of repeated T-DNA units to produce silencing. Although the PCR assay used here is not absolutely quantitative, it does suggest that the average transgene dosage is about 2 implying that some of the lines exhibiting suppression have single insertions. In several of our lines, the suppression obtained is profound (Figure 2) which makes this strategy even more attractive to those interested in specifically switching off gene expression. There is one previous report of the deliberate combination of repetitive DNA with a reporter gene effecting increased HDGS: Lohuis et al., Plant Journal, 8, 919-932 (1995) inserted a copy of a randomly isolated repetitive genomic sequence (RPS) upstream of GUS reporter gene and found that this element increased the frequency of variegation of

20-04-2005

Printed: 22-04-2005 TIPA 99910592

transgene expression. This is an example of cis-inactivation, probably acts at the transcriptional level, and the authors considered it to be distinct from co-suppression/sense-suppression phenomena. Interestingly, the RPS element did not increase the frequency of complete silencing of the transgene. In our example, although the level of suppression is severe in many lines, it is not possible to say whether the degree of suppression is equal in all cells expressing the target gene or if the repeat has simply greatly increased the proportion of cells experiencing suppression.

Example 9

Constructs and transformation

The tomato ACO1 cDNA, pTOM13 was released from its original cloning vector, pAT153, (Promega), creating pG31. pG31 was digested with EcoRI and the vector religated to create pTRD. This removed the 5' end of the cDNA which contains approximately 90 base pairs of the 3'untranslated region in the antisense orientation at its 5' end which may have been introduced artefactually during the original cloning of the pTOM13 cDNA . The remaining ACO1 sequence was cut out from pTRD with EcoRI and HindIII and ligated into $pT_7.T_{3\alpha}18$ (BRL) digested by EcoRI and the ends filled in with Klenow enzyme. The 5' untranslated region of the ACO1 transcript (minus approximately 10 bases at the 5' end) was amplified with Taq polymerase from oligo dT-primed cDNA of wounded tomato leaves with the primers 5' CATTCATCTCTTCAATCTTTTG 3' (SEQ ID No.2) and 5' CTTAATTTCTTGGTAAAGTGTTTTCC 3' (SEQ ID NO.3). This DNA was rendered flush ended with T4 DNA polymerase and ligated with the filled in pTRF to create pMI1. This reconstituted the EcoRI site at the 5' end and yielded a translatable ACO1 cDNA slightly shorter than the wild type ACO1 mRNA. Sequencing confirmed that the amplified ACO1 sequence was not mutated. pMI1 was digested with HindIII and partially with EcoRI and the fragment containing the ACO1 cDNA sequence was filled in with Klenow enzyme, and ligated with SmaI digested pDH51 to create pDHC1. This was digested with XbaI and HindIII, the filled in and the fragment containing the vector, 35S promoter and ACO1 cDNA religated to create pMI5. pMI7 contains two copies of the 5'UTR of ACO1 tandemly linked and inserted in the antisense orientation upstream of the 5'UTR of ACO1 in pMI5. This was

508 20-04-2005

Printed: 22-04-2005 TIPAT 99910592

made by amplifying the 5'UTR from tomato leaf cDNA (see above) with oligos 5' CATTCATCTCTTCAATCTTTTG 3' and 5' CTTAATTTCTTGGTAAAGTGTTTTCC 3', polishing the DNA with T4 DNA pol and ligating it into a filled in Acc651 site in pMI5 upstream of the 5'UTR of the ACO1 sequence Acc651 (an isoshizomer of Kpn1 but which gives a 5' overhang). The construction was confirmed by sequencing.

pDHC1 and pMI7 were digested with BamHI, BglI and PvuII and the BamHI-PvuII fragments containing the CamV35S-ACO1cDNA sequences were cloned into Bin19 which had been cut by HindIII, filled in and then cut by BamHI. The resulting recombinants were called pBC1 and pBM17 respectively. These plasmids were transformed into A.tumefaciens LBA4404: and this used to transform tomato cotyledons (*Lycopersicon esculentum* var Ailsa Craig). Plants were regenerated from callus grown on 50$\mu g.ml^{-1}$ kanamycin.

Example 10

ACC-oxidase assays

ACC-oxidase activity was measured as the ability of plant tissue to convert exogenous 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene. Discs were cut from leaf lamina with a sharp cork borer and placed in contact with 0.5ml of 10mM NaH_2PO_4/Na_2HPO_4 (pH7), and 10 mM ACC (Sigma) in 5 ml glass bottles which were then sealed with "Subaseal" vaccine caps (Fisons). After 1 hour at room temperature, the ethylene in the head space was measured by gas chromatography as described by Smith et al., 1986. Ethylene was also measured from bottles containing the solution but without leaf tissue. These values were subtracted from the values obtained from the bottles containing leaf discs.

Example 11

PCR analysis of transgenic plants

DNA was extracted from singles leaves of wild type plants, plants homozygous for a ACO-antisense gene, and those transformed with the constructs of pBC1 and pBM17. Leaves were frozen in liquid nitrogen, briefly ground in eppendorf tubes with a disposable pipette tip, ground further after the addition of 200µl DNA extraction buffer (1%

509 20-04-2005

22-04-2005
TIPA
99910592

laurylsarcosine, 0.8% CTAB, 0.8M NaCl, 0.02M EDTA, 0.2M Tris/HCl (pH8)), heated to 65°C for 15 minutes, extracted once with phenol/chloroform and the DNA precipitated from the aqueous phase by the addition of 0.6 volumes of isopropanol. The DNA was recovered by centrifugation, the pellets washed in 70% ethanol, dried and redissolved in 200ul, of TE buffer. 1ul of this was used as template for simultaneous PCR amplification of the endogenous ACO1 gene and the transgene using the primers ACO1.1 (ATGGAGAACTTCCCAATTATTAACTTGGAAAAG) and the ACO1.2 (CTAAGCACTTGCAATTGGATCACTTTCCAT) for 21 cycles of 30 seconds at 95°C, 30 seconds at 65°C and 1 minute at 72°C. Amplified DNA was separated by electrophoresis in a 0.8% agarose/1xTBE gel and blotted onto HybondN+ in 0.4M NaOH for 6 hours. To detect the amplified ACO sequences, the DNA on the filter was hybridised with random prime labelled ACO1 cDNA. The filter was washed in 0.2xSSPE/1%SDS at 65°C followed by phosphorimaging of the radioactive signal.

Example 12

Treatment of leaves with cycloheximide and mechanical wounding

Compound leaves were excised with a sharp scalpel blade and immediately placed under water solution of 50µl.ml^{-1} cycloheximide (Sigma). Another 3 cm of the stalk was cut from the branch under the solution and the assembly was then left in a laminar airflow for six hours to allow the cycloheximide to enter the leaves.

To wound leaf tissue, individual leaflets were placed on a hard surface and diced with a sharp scalpel blade approximately 10 times transversely and 5 times longitudinally.

Example 13

Northern analysis of ACO mRNA in leaves treated with cycloheximide

RNA was extracted from cycloheximide treated leaves as follows. Tissue was frozen in liquid nitrogen and pulverised either in a coffee grinder (for fruit pericarp, see below) or in a mortar (for leaves). 5ml.gfwt^{-1} of RNA extraction buffer (Kirby's) was added and the frozen slurry ground further in disposable polypropylene centrifuge tubes with a glass rod.

20-04-2005

Once thawed, the mixture was extracted twice with phenol/chloroform and the nucleic acids precipitated by the addition of 2.5 volumes of ethanol, 1/10 volume 3M sodium acetate (pH5) and refrigeration at 20°C for 1 hour. After centrifugation at 3000xg for 10 minutes (40 minutes for a fruit extraction), the pellets were redissolved quickly in water (approximately 1ml per gram of tissue) and, an equal vol. of 2x DNA extraction buffer (1.4M NaCl, 2% CTAB, 100mM Tris/HCl (pH8)). Two volumes of precipitation buffer (1%CTAB, 50mM Tris/HCl (pH8)) were added to precipitate the nucleic acids (30 minutes at room temparature suffices) and the precipitate was collected by centrifugation (3000xg/15 minutes). This step was repeated except the pellets were dissolved in 1xDNA extraction buffer. After collection of the second precipitation, the pellets were redissolved in 0.5ml 1M NaCl and immediately reprecipitated with 2.5 volumes of ethanol (-20°C/30 minutes). After centrifugation (10000xg/10 minutes), the pellets were redissolved in 400µl water and extracted twice with phenol/chloroform. The nucleic acids were precipitated and collected as above redissolved in 400µl water. 46ul of 10 x One-Phor-All-Buffer (Pharmacia) was added with 50 units of RNAase-free DNAase (Promega) and the solutions incubated at 37°C for 30 minutes. They were extracted twice with phenol/chloroform, the RNA precipitated and collected as above and finally redissolved in 100-500ul of water. We have found that this relatively extensive purification is necessary if rare transcripts are to be detected by RPA. Also, the RNA redissolves readily which greatly reduces handling time when manipulating this RNA mixed with radioactive probe RNA.

50µg of leaf RNA was mixed with an equal volume of denaturation/loading solution (50% formamide; 25mM sodium phosphate (pH6.5); 10mM EDTA; 6.2% formaldehyde; 200µg.ml^{-1} ethidium bromide) and separated by electrophoresis on a 25mM sodium phosphate (pH6.5) /3.7% formaldehyde /1.5% agarose gel in 10mM sodium phosphate (pH6.5)/3.7% formaldehyde with continuous buffer recirculation. The separated RNA was blotted onto Genescreen (Dupont) hybridisation membrane in 10mM sodium phosphate (pH6.5). The autocrosslink setting on a Stratalinker (Stratagene) was used to covalently link the RNA to the filter. The filter was prehybridised and then hybridised with a 32P-random prime labelled ACO1 cDNA probe. The filter was washed in 0.2xSSPE/1%SDS at 65°C and then exposed

22-04-2005 TIPA 99910592

to Kodak X-omat film between two intensifying screens at -70 for 24 hours. Subsequently the radioactivity in each band was measured by phophorimaging.

Example 14

Ribonuclease protection analysis

RNA was extracted from cycloheximide treated leaves and fruit described above.

RNA probes were transcribed with T7 RNA polymerase at 20°C with α-^{32}P UTP (400Ci. mmol^{-1}) as the sole source of UTP. After 1 hour incubation, RNAase-free DNAase was used to remove the template and the probe was further purified on 6%polyacrylamide/8M urea/1xTBE gels. The band containing the full length probe was visualised by autoradiography. The gel slice containing this RNA was excised and placed in 1ml probe elution buffer (0.5M ammonium acetate; 1mMEDTA; 0.2% SDS) for between 6 and 14 hours at 37°C. Typically, between 20m and 100µl of this would be co-precipitated with between 20 or 100µg of the RNA to be tested plus two yeast RNA controls. The precipitated RNAs were redissolved in 30µl hybridisation solution (80% formamide; 40mM PIPES/NaOH; 0.4M sodium acetate; 1mM EDTA pH should be 6.4) heated to 65°C for 10 minutes and hybridised at 42°C for between 2 to 14 hours. The longer hybridisation times were purely for convenience since we easily detected even rare transcripts after only 2 hours of hybridisation. 300µl of RNAase digestion buffer (5mM EDTA; 200mM sodium acetate; 10mM Tris/HCl. Final pH of solution should be 7.5) containing either RNAaseONE (Promega) or RNAase T1 (Ambion) was added to each tube except one containing yeast RNA which received RNAase digestion buffer without any ribonuclease. Incubation of the digesting RNA was at either 25°C (RNAaseONE) or 37°C RNAaseT1) for 2-4 hours. RNAaseONE was inactivated by the addition of SDS to 0.5% and the protected, double stranded RNAs were precipitated with ethanol and sodium acetate. RNAaseT1 was inactivated and the double stranded RNAs were precipitated by the addition of the inactivation/precipitation solution provided with the RNAase protection kit from Ambion. The protected RNAs were redissolved in 5-10ul of denaturation/loading solution (80% formamide; 10mM EDTA; 0.1% bromophenol blue; 0.1% xylene cyanol; 0.1% SDS), heated to 95°C for 5 minutes and then sepated by electrophoresis on a on 6-8% polyacrylamide/8M urea/1xTBE gels (the concentration of polyacrylamide

20-04-2005

depending on upon the sizes of the fragments to be separated). After electrophoresis, the gels were dried and exposed to Kodak x-omat film between two intensifying screens at -70 for the time indicated. The radioactivity was measured by phosphorimaging.

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO: 1 (EMBL Accession No. X58273)

SQ Sequence 3681 BP; 1153 A; 564 C; 682 G; 1282 T; 0 other;

```
AAATTTGATA GATTCAGTTT TTATGTTTTT AGTGCTGATT ACAACATTGA AATTCTAAAT      60
TTAGAATTTA ATATTTATTA AATGTTAGTG CATTTATACA AATAACATAT TACATCTCAA     120
ATAATATTGA GTTTGTTAGA TTTTATTTGC CCTGATTTCT TATCATAAAT AGGTTTTCCT     180
TTTAGGAAAA GGTTTTGAAT TGACTATTCT TTTTTTGGTA GGAAAAAGTT TAGGACTCTA     240
TAAATAGAGG CATGTTCCTT CTAACTTAAT TAGCATTCAC AATGTAGTTT TAAGGGCTTT     300
GAGAGTTTTG GTTAGAGGGA GAATTTGTGA ACCTCTCATG TATTCCGAGT GAATTGGTTG     360
AGGTTGTTTC CCTCTGTATT TTGTACTCTC ATGTTTATAG TGGATTGCTC ATTTCCTTTG     420
TGGACGTAGG TCGATTGACC GAACCACGTT AAATTTTTGT GTCTTTTGGT ATATTTCCTG     480
TTCTTCTTAC TCGTGGTCTT TCGAGGTTTG CTTTGCTAGC TTCCGCGTTT ACACCTGCTT     540
ATTTTCGGTC CTAACAAGTG GTATCAGAGC CAGATTCAAT AATGGAGTCA GGTGTAGTGG     600
TTCGATAATC GATGATTGAA CCAAGTTAGA AAGAGGTGTT CATCTTGACG GGTGTAGTTC     660
TAGCCGCAAC CTTTTTGACA GTAATGAAGA TTTTGATGGA GAAATTGTTT CAGAGAGGTT     720
CTCTGTGTTG AGACATAAAT TTTGTAAAGG AGATTATGGA GAGGAGAAGC AAGTTGTTGA     780
AGATTAAGTA AAGAAGGTGG ACAAATCTAT TTTGTCAGAA ATTCAGGCCA AGGGGGAGAT     840
TTGTTGGGTT TTATTTGCCC TGATTTTTTA CCATAAATAG GTTTTCCTTT AAGGAAAAGG     900
TTTTGAATTG ACTATTCTTT TTTTGGTAGG AAAAGGTTTA GGATTCTATA AATAGAGGCA     960
TGTTCCTTCT AACTTAATTA GCATTCACAA TGTAGTTTTA AGGGCTTTGA GAGTTTTGGT    1020
TAGAGGGAGA ATTTGTGAAC CTCTCATGTA TTCCGAGTGA ATTGGTTGAG GTTGTTTCCC    1080
TCTGTATTTT GTACTCTCAT GTTTATAGTG GATTGCTCAT TTCCTTTGTG GACGTAGGTC    1140
```

Printed: 22-04-2005 TIPAT 99910592

GATTGACCGA ACCACGTTAA ATCTTTGTGT CTTTTGGTAT ATTTCTCGTT GTCTTCTTAC 1200

TCGTGGTCTT TCGAGGTTTG CTTTGCTAGC TTCCGCGTTT ACACCTGCTT ATTTGCGGTC 1260

CTAACAGAGT TCGATGGGTT GAATCTATAA AAAGAAAAAT ATACTCGTGA TTCACGATTA 1320

TTTATATGAA AATATAATAA ATATTGAATT TCCTTTGCTA TTTCTTATGT TTACGTCTTT 1380

ATATTTCAAA TTATTCCACC AATACTGACA AGCCCTAGGC CATCTCTAGG AAATTCATAC 1440

AATTTTTTTT TTGTTGTTAA CTAGTTAAAT TGGCAGCCTT AAAGATTATT GTAAAATTCA 1500

AGGCAACTTC CTCAAGTACT ACAACTACAT TGTAACATCC CAGTCAAAGT GTCCTAAAAT 1560

TTTATAAAAT TTGACACATG AAACAATAGC ACAATAAATT TTAGTACTAT TGCAGCCATG 1620

GCCCATAAGC CATCATGTAT TATAGTCAAA ATGGGTCCTT TTCCAATTTG TCTTGATCCC 1680

AAAATCCCTT TGTAGGTAAG ATGGTTCAAC AAGGAACTAT GACTCTTAAG GTAGACTTGG 1740

ACTCATAGAC TTGTCATAAC TCATAAAGAC TTGGAATATA ATAATTATTC ATTTAAATTA 1800

TAATTCTCTA CTTTAATATC TTCTACTATA AATACCCTTT CAAAGCCTCA TTATTTGTAC 1860

ATCAAACATT GATATTCATC TCTTCAATCT TTTGTATTCA CATATTCTAT TTATTCAATA 1920

CACTTAGGAA AACACTTTAC CAAGAAATTA AGATGGAGAA CTTCCCAATT ATTAACTTGG 1980

AAAAGCTCAA TGGAGATGAG AGAGCCAACA CCATGGAAAT GATCAAAGAT GCTTGTGAGA 2040

ATTGGGGCTT CTTTGAGGTA ATCATAAATT ACATAAACAT ATTAATATGT TTGTTTCAAT 2100

TTATCAGTCA TACTTTTCTC TGTTTTAAAA TTAATGTCAC TTTCAATATT TAATAATTCG 2160

CATGACATGT TTATAACACA ACAAGATATA GGTTACATTT TGATACATTA TATATAACTT 2220

CTGTCACACG ACTCAAAAGT CTTTCTTAAT TTCTTGAATT CAATGATCGA TCAAACTAAG 2280

ACACGTAAAA TGAAACGGGG AATAGTAATT CTGTTTGCTT ATGTGATCAT TGTAGTTGGT 2340

GAACCATGGA ATTCCACATG AAGTAATGGA CACAGTAGAG AAAATGACAA AGGGACATTA 2400

CAAGAAGTGC ATGGAACAGA GGTTTAAGGA ACTAGTGGCA AGTAAGGGAC TTGAGGCTGT 2460

TCAAGCTGAG GTTACTGATT TAGATTGGGA AAGCACTTTC TTCTTGCGCC ATCTTCCTAC 2520

TTCTAATATC TCTCAAGTAC CCGATCTTGA CGAAGAATAC AGGTACATAC ATGTGTCCTA 2580

CATATTGCGT ATATAATAAA TAAACACAAA ATTTAAGTTA TATACGCTGA CAGTATAACT 2640

20-04-2005

AATTATAATG TTGTACCAAA TGATGCAGAG AGGTGATGAG AGATTTTGCT AAAAGATTGG 2700

AGAAATTGGC TGAGGAGTTA CTTGACTTAC TCTGTGAAAA TCTTGGACTT GAAAAAGGTT 2760

ACTTGAAAAA TGCCTTTTAT GGATCAAAAG GTCCCAACTT TGGTACTAAA GTTAGCAACT 2820

ATCCACCATG TCCTAAGCCC GATTTGATCA AGGGACTCCG CGCTCATACA GACGCAGGAG 2880

GCATCATACT TCTGTTCCAA GATGACAAAG TGAGTGGCCT TCAACTCCTC AAAGACGAGC 2940

AATGGATCGA TGTTCCTCCC ATGCGCCACT CTATTGTGGT TAACCTTGGT GACCAACTTG 3000

AGGTACAAGA TTCACTAAGT GTGTGTGTTT TTATCACTAT AACTTAGAAG TAGTAACTAA 3060

AAATGGTATT AATGAAATGT TATAAAAACA GGTGATCACT AACGGGAAGT ACAAGAGTGT 3120

GCTGCACAGA GTAATTGCAC AAACAGACGG GACACGAATG TCATTAGCCT CATTTTACAA 3180

TCCAGGAAGT GATGCAGTAA TATATCCAGC AAAAACTTTG GTTGAAAAAG AGGCAGAGGA 3240

AAGTACACAA GTGTATCCAA AGTTTGTGTT TGATGATTAC ATGAAGTTAT ATGCTGGACT 3300

CAAGTTTCAA GCCAAAGAGC CAAGATTTGA AGCAATGAAG GCAATGGAAA GTGATCCAAT 3360

TGCAAGTGCT TAGATCCCAA TTCAATTAAA AAAATTGGTG TTTGAAAAAT ATATTTAAAT 3420

ATAGCAATCT ATGTATACAC ATTATTTGCT CTTCTTATGT ATGGTAGAAT AAAGTTAGTA 3480

TTAAAAAAGA TTGTGATTTG CTGCATATGT ATCAAAAAGA GTCCTAATAT TTGTATCTAT 3540

AAATAAGGTG CCTTCTAGTG AAATTATACA AATAATAATT TGGAGTGTAT TGTTCTTTCT 3600

CATGTAATTT AACTTTTAAG TATCTTACTT TACAATATAC TGTTCACTTA TTGAACATAT 3660

TGAGTGATAT ATTGACTCAA T 3681

FILE COPY 22-04-2005

TIPAT

99910392

CLAIMS

1. A method of controlling the expression of a DNA sequence in a target organism, comprising inserting into the genome of said organism a gene construct comprising in sequence a promoter region, a 5'-untranslated region, a transcribable DNA sequence and a 3'-untranslated region containing a polyadenylation signal, characterised in that the said construct includes an inverted repeat of a fragment of said construct.

2. A method as claimed in claim 1 in which the inverted repeat is a fragment of the 5'-untranslated region of the said construct.

3. A method as claimed in claim 1 or claim 2, in which the inverted repeat is separated from the selected fragment by a sequence of nucleotides acting as a spacer.

4. A method as claimed in claim 1 or 2 or 3, in which the construct includes a double copy of the inverted repeat.

5. A method as claimed in any preceding claim in which the construct has a selected fragment fused to two copies in tandem of the inverted repeat.

6. A DNA construct for the inhibition of gene expression comprising in sequence a promoter region, a 5'-untranslated region, a transcribable DNA sequence and a 3'-untranslated region containing a polyadenylation signal, characterised in that the said 5'-untranslated region is followed immediately by a pair of tandem inverted repeats of said 5'-untranslated region.

516

20-04-2005

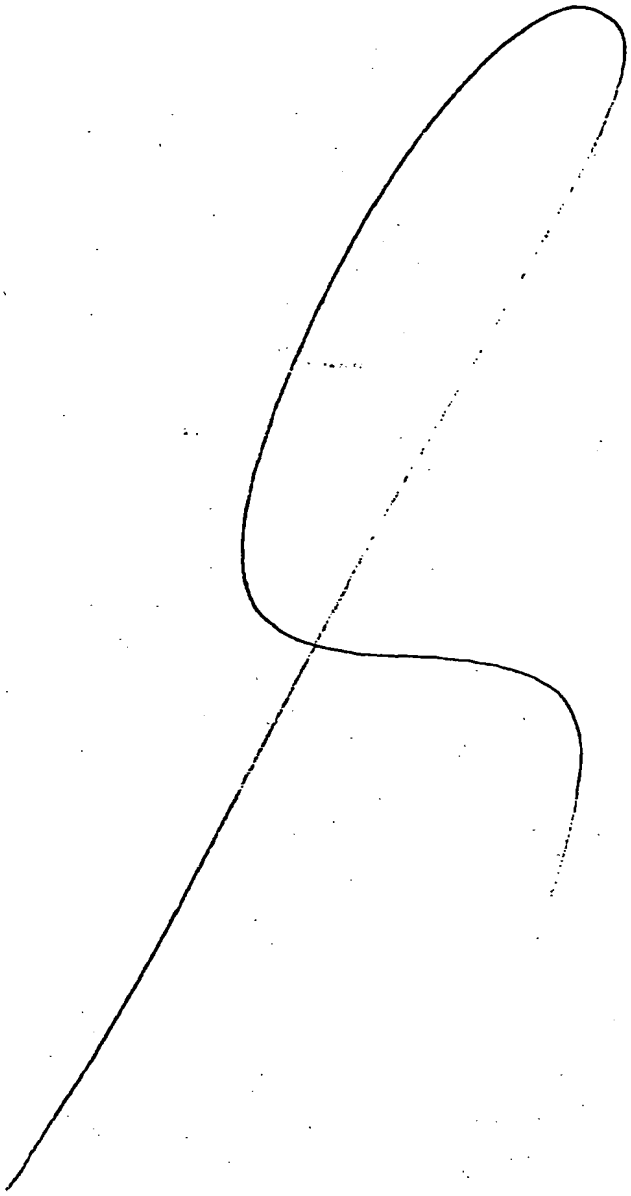
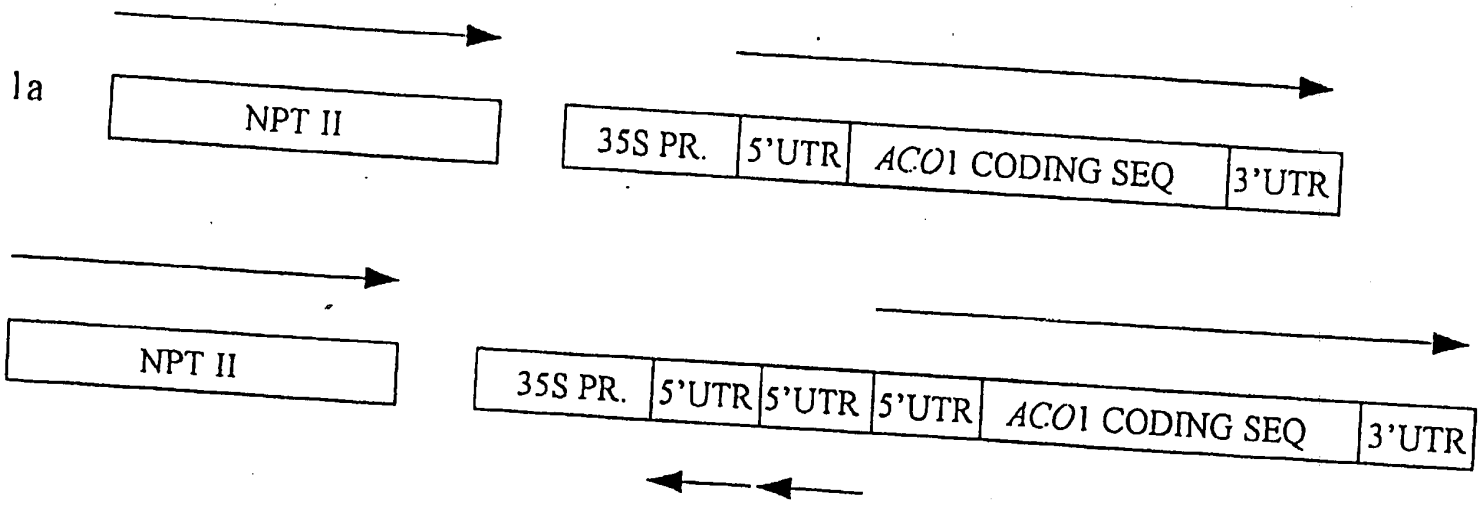
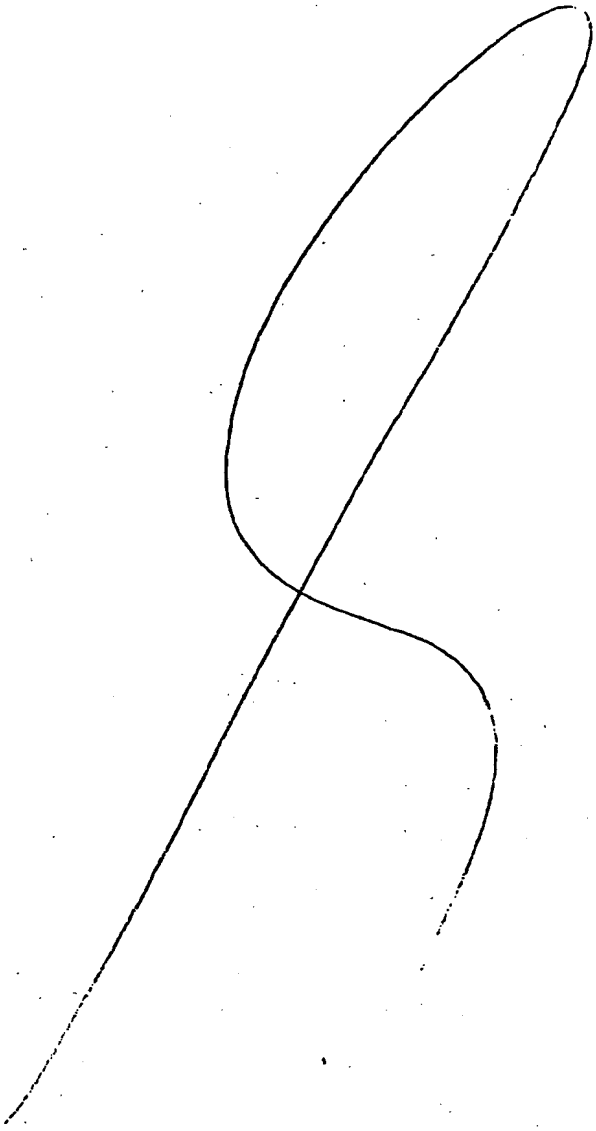
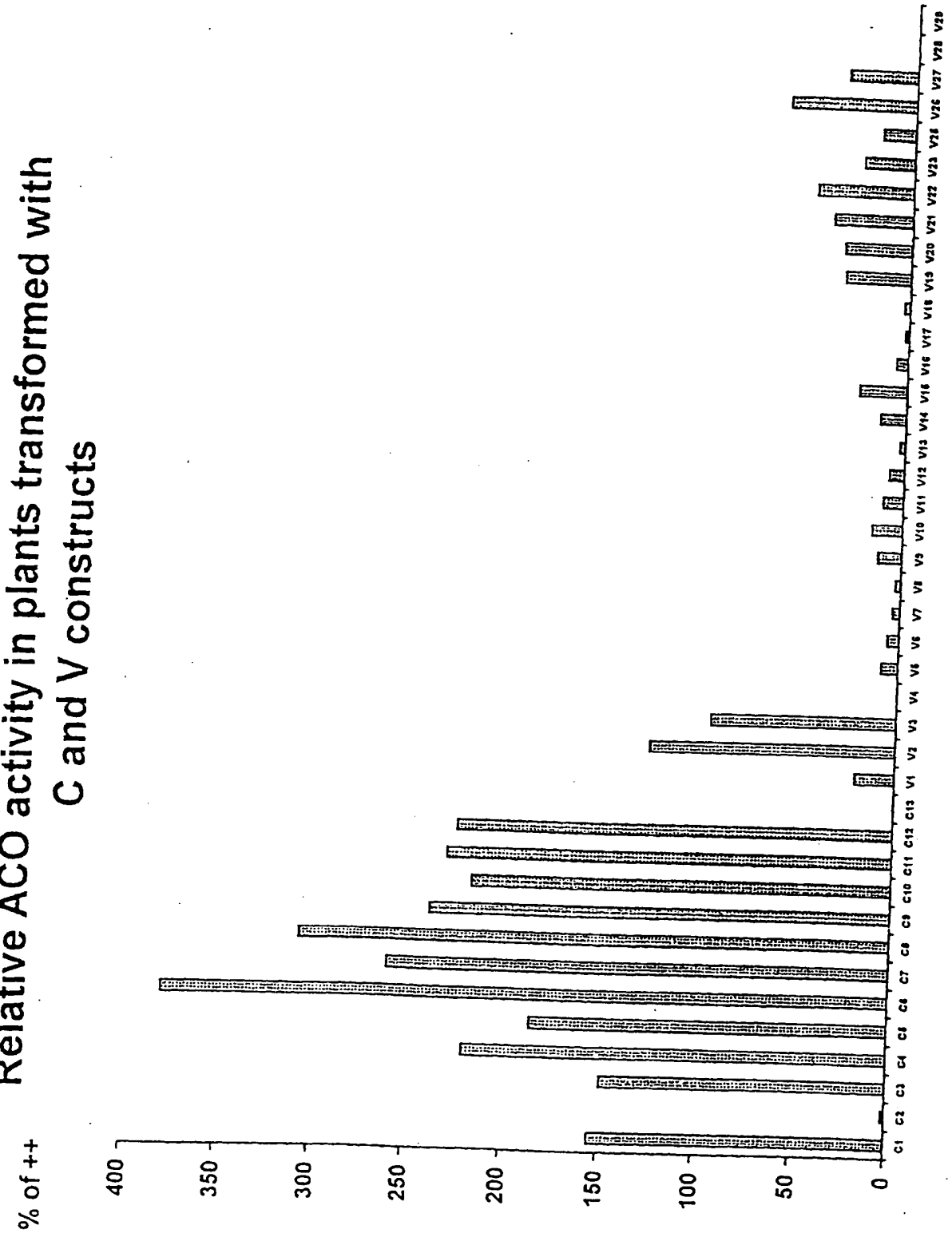


FIGURE 1


1a
NPT II
35S PR.
5'UTR
ACO1 CODING SEQ
3'UTR
C
NPT II
35S PR.
5'UTR
5'UTR
5'UTR
ACO1 CODING SEQ
3'UTR
V




Relative ACO activity in plants transformed with C and V constructs
% of ++
400
350
300
250
200
150
100
50
0
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 V1 V2 V3 V4 V5 V6 V7 V8 V9 V10 V11 V12 V13 V14 V15 V16 V17 V18 V19 V20 V21 V22 V23 V24 V25 V26 V27 V28

ated: 22-04-2005

TIPAT

99910592

PCT/GB98/01450

ZENECA AGROCHEMICALS

20 MAY 1998

521

20-04-2005

THIS PAGE BLANK (USPTO)

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☑ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☑ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)